MITOCHONDRIAL TRANSPORT AND PROCESSING OF METHYLMALONYL COENZYME A MUTASE IN CULTURED BUFFALO RAT LIVER CELLS

DAVID C. HELFGOTT

1983

Permission for photocopying or microfilming of "

MITOCHONDRIAL TRANSPORT AND PROCESSING OF

(title of thesis)

METHYLMALONYL COENZYME A MUTASE IN CULTURED BUFFALO RAT LIVER CELLS"

for the purpose of individual scholarly consultation or reference is hereby granted by the author. This permission is not to be interpreted as affecting publication of this work, or otherwise placing it in the public domain, and the author reserves all rights of ownership guaranteed under common law protection of unpublished manuscripts.

David Helfgott

(Signature of author)

DAVID HELFGOTT

(Printed name)

3/1/83

(Date)

MITOCHONDRIAL TRANSPORT AND PROCESSING OF METHYLMALONYL COENZYME A MUTASE IN CULTURED BUFFALO RAT LIVER CELLS

David C. Helfgott

B.A., University of Pennsylvania, 1978

A thesis Submitted to the Faculty
of the Yale University School of Medicine
in Partial Fulfillment of the Requirements for the degree of
Doctor of Medicine

1983

For my parents, with love and appreciation

ACKNOWLEDGEMENTS

Since I began working on this project in the summer between my first and second years of medical school, many people have contributed to its success and to the enjoyment I have derived from it. I especially want to express my sincere thanks to Dr. Leon Rosenberg; he has been supportive throughout these last three years and was very patient with my slow progress during the time-consuming clinical years. More importantly, he has been a great teacher, an example to follow, and most of all, a friend. I wish him all the best in the future.

I am also grateful to Dr. Wayne Fenton, who guided me through this project and made the lab a much more pleasant and interesting place to work.

Many others made it fun to keep coming back. Among them are Adele Hack, who also provided essential technical advice and assistance, Fleming, Jan, Frante, Michelle and Alda.

This thesis rounds out a great education which I have received at Yale Medical School. I must express my appreciation and thanks to those people who have made these last four years enjoyable and completely worthwhile. In particular, I am deeply grateful to Dr. Vincent T. Andriole, not only for his positive influence and superb teaching, but also for just understanding. He is a great individual and a great clinician who has made an indelible impression on me. Thanks.

TABLE OF CONTENTS

ABSTRACT

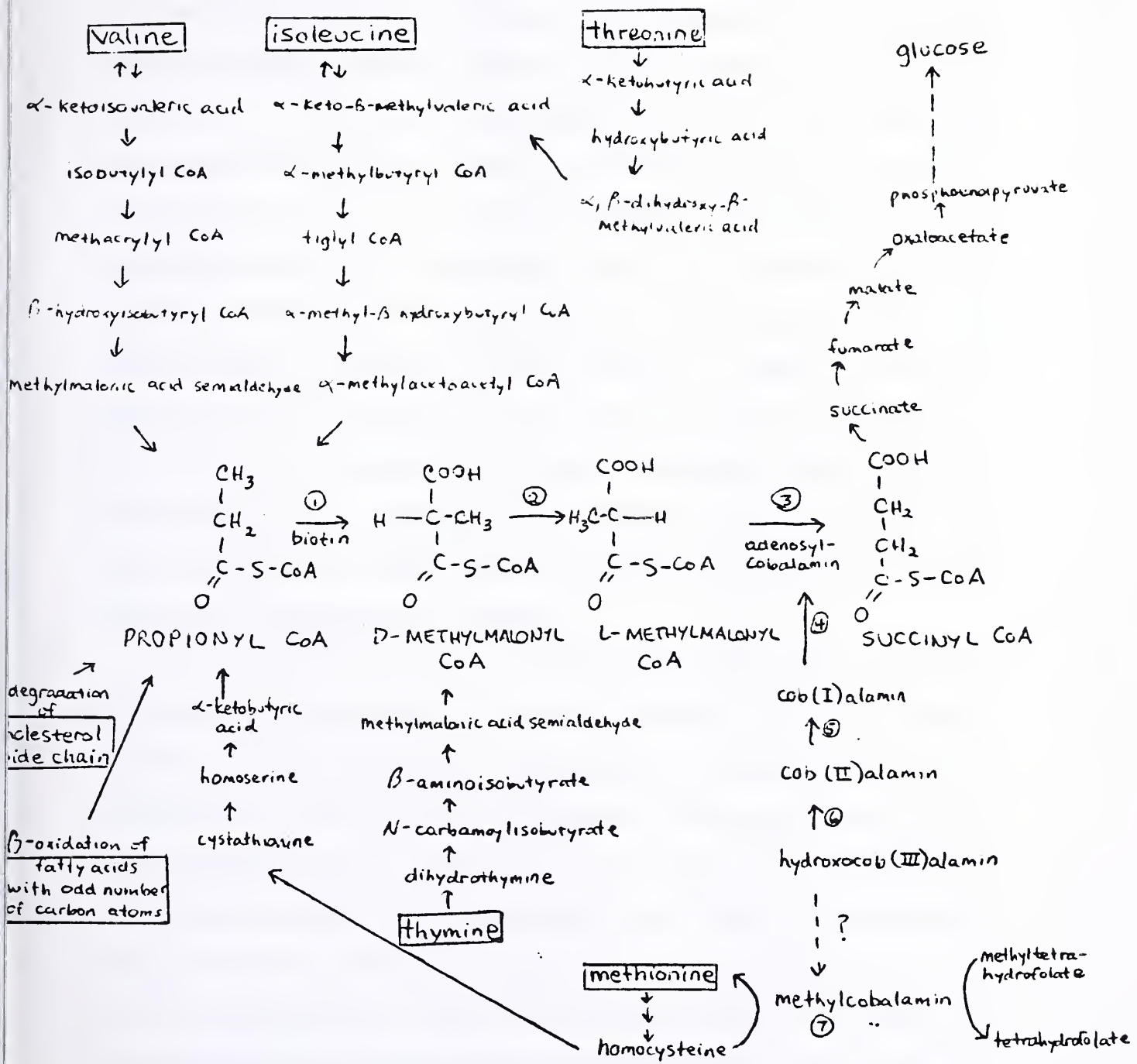
Methylmalonyl coenzyme A mutase, a cytoplasmically synthesized mitochondrial enzyme, catalyzes the conversion of L-methylmalonyl coenzyme A (CoA) to succinyl CoA. Methylmalonic acidemia is an inborn error of metabolism which is characterized by derangements in the activity of this enzyme, secondary either to defects in the synthesis of its cofactor, adenosylcobalamin, or to defects in the structural integrity of the enzyme itself. The work presented here shows that, like many other previously studied cytoplasmically synthesized mitochondrial proteins, methylmalonyl CoA mutase is synthesized first as a precursor which is subsequently processed by the mitochondrion to the mature form. This maturation can be blocked by 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation which deprives the mitochondrial inner membrane of energy. The conversion of precursor to mature methylmalonyl CoA mutase subunit takes place with a $t_{1/2}$ of 6-9 minutes. In contrast to the few reports published for other proteins, the precursor form of mutase is not rapidly degraded if maturation is inhibited. The implications of these findings are discussed with respect to the biosynthesis of mitochondrial proteins made in the cytoplasm and with respect to the possible molecular defect in the *mut*^{o} mutant group of methylmalonic acidemia.

INTRODUCTION

Propionate formed by bacterial fermentation is a major source of energy in ruminants. In non-ruminants, propionate is found mostly as the coenzyme A (CoA)[1] ester, and is a product of the catabolism of the amino acids valine, isoleucine, methionine and threonine, with a smaller contribution of propionate resulting from the beta-oxidation of fatty acids with an odd number of carbon atoms. A small amount of propionate is formed from the catabolism of cholesterol. (1) The propionate formed by these processes is converted to succinate through a sequence of reactions which involves first the carboxylation of propionyl CoA to D-methylmalonyl CoA, then the racemazation of D-methylmalonyl CoA to the L-isomer and finally the isomerization of L-methylmalonyl CoA to succinyl CoA (2,3). The catabolism of thymine produces D-methylmalonyl CoA, which similarly is metabolized to succinyl CoA (1). The conversion of L-methylmalonyl CoA to succinyl CoA is catalyzed by the enzyme methylmalonyl CoA mutase (E.C. 5.4.99.2) in the presence of a vitamin B_{12} coenzyme, adenosylcobalamin. The succinyl CoA formed can enter the tricarboxylic acid cycle to be converted to carbohydrate. The major pathway of propionate metabolism is illustrated in more detail in figure 1.

[1]Abbreviations used throughout this thesis: CoA, Coenzyme A; AdoCbl, adenosylcobalamin; OH-Cbl, hydroxocobalamin; OTCase, ornithine transcarbamylase; CPS, mitochondrial carbamyl phosphate synthetase; CCCP, carbonyl cyanide m-chlorophenylhydrazone; DNP, 2,4-dinitrophenol.

Figure 1. The major pathway of propionate metabolism is shown in the center of the figure. The entrance of propionyl CoA and methylmalonyl CoA precursors into the pathway is also pictured. At the bottom right of the figure a simplified version of cobalamin metabolism is shown. The important enzymes, most of which will be referred to later in the text, are included.



① propionyl CoA carboxylase
② methylmalonyl CoA racemase
③ methylmalonyl CoA mutase
④ cob(I)alamin adenosyltransferase
⑤,⑥ reductase(s)
⑦ N^5-methyltetrahydrofolate methyltransferase

Figure 1. (legend on preceding page)

Methylmalonyl CoA mutase is composed of 2 identical ~75,000 dalton subunits, each of which binds one molecule of cofactor to form the holoenzyme (1,4,5). Although the isomerization reaction which it catalyzes is reversible, conditions in the cell greatly favor the conversion of methylmalonyl CoA to succinyl CoA (4). Studies of the reaction itself have demonstrated that it involves the intramolecular transfer of the coenzyme A carboxyl radical rather than the transfer of the free carboxyl group (6,7). It is one of two enzymes in higher organisms which require the presence of a vitamin B_{12} (cobalamin) coenzyme; the other is methyltetrahydrofolate methyltransferase, which uses methylcobalamin as cofactor.

The requirement of a cobalamin coenzyme for the conversion of propionyl CoA to succinyl CoA was first confirmed in 1959 by Smith and Monty (8). They measured ^{14}C incorporation into succinic, fumaric, and malic acids when liver homogenates of vitamin B_{12} deficient and vitamin B_{12} supplemented rats were incubated with $^{14}CO_2$ and propionyl CoA. In the two groups of rats there was no difference in the ^{14}C incorporation when the incubation was performed in the presence of excess mutase prepared from sheep kidney. However, without added mutase, significantly less succinate, malate, and fumarate were formed by the homogenates from the vitamin B_{12} deficient rats compared to those from the supplemented group. In addition, the radioactivity recovered as methylmalonic acid was similar for the two

groups, supporting the conclusion that the cobalamin was necessary for the conversion of methylmalonyl CoA to succinate (8). Almost simultaneously, Gurnani and coworkers (9) identified the active cofactor form by adding adenosylcobalamin to extracts of mitochondria from vitamin B_{12} deficient rat liver incubated with propionyl CoA and $NaH^{14}CO_3$. This greatly increased the incorporation of $^{14}CO_2$ into succinate in the presence of propionyl CoA. Other cobalamin analogues would not support the increased synthesis of labeled succinate. Finally, using ox liver extracts, Stern and Friedman (10) showed that the conversion of methylmalonyl CoA to succinate was enhanced by the addition of adenosylcobalamin.

Briefly. vitamin B_{12} is synthesized by microorganisms and found in the tissues of plants and animals. It consists of a planar corrin ring surrounding a central cobalt atom, with a side group extending down from the plane composed of a 5.6-dimethylbenzimidazole group, a ribose molecule and a phosphate group (1). Extending up from the corrin plane and attached to the cobalt atom may be a variety of moieties which characterize the specific cobalamin. It may be a cyanide radical, common in commercial preparations of the vitamin but not occurring naturally, or forms which are found in animals, such as a hydroxyl, methyl, or adenosyl group. In the latter two cases, mentioned above in reference to their coenzyme function, the cobalt atom is reduced to the +1 valence state. In hydroxo- or

cyanocobalamin, the forms delivered to the mammalian cell by the peripheral circulation, the cobalt atom exists in the +3 oxidation state.

Ingested vitamin B_{12} is transported across the terminal ileum bound to a gastric glycoprotein (intrinsic factor) recognized by specific receptors on the ileal mucosal cells. The vitamin moves into the bloodstream where a beta-globulin transport protein, transcobalamin II, binds it and delivers it to specific tissues. Receptor-mediated endocytosis of the vitamin-carrier complex, fusion of the endocytic vacuole with a lysosome, and proteolytic degradation of the transcobalamin II transports the cobalamin into the cytosol. (1,11)

Cobalamin which has entered the cell must be reduced from the +3 (cob(III)alamin) to the +1 (cob(I)alamin) oxidation state which characterizes the active methyl- and adenosylcobalamin analogues. The events leading to the formation of methylcobalamin are not well described, although it is known that the methyltransferase enzyme is localized to the cytosol (12). Mutase. however, is a mitochondrial enzyme (13), and the latter steps of the reduction sequence occur in the mitochondrial compartment (14). This scheme has been worked out in bacteria (15) and has been supported using mammalian cells (14). As shown in figure 1, cob(III)alamin is reduced by flavoprotein reductases to cob(II)alamin and then to cob(I)alamin before adenosyltransferase adds an adenosyl group from ATP to form

the active cofactor for mutase.

The details of methylmalonyl CoA mutase synthesis, compartmentalization, and properties are the subject of this study and will be discussed later. In addition to providing insight into the details of cellular enzyme kinetics and compartmentalization, the study of methylmalonyl CoA mutase is integral to understanding the methylmalonic acidemias.

The Methylmalonic Acidemias

An abnormally high concentration of methylmalonic acid in the blood and urine may be caused by a variety of clinical entities, including nutritional vitamin B_{12} deficiency, pernicious anemia (16,17), and methylmalonic acidemia. Methylmalonic acidemia (or aciduria) is not a single entity as once thought, but rather describes a group of biochemical defects which reflect inborn errors of metabolism and which are manifested by derangement of the action of methylmalonyl CoA mutase. The clinical hallmark of the disease is the extremely high urinary excretion of methylmalonic acid and its presence in significant concentration in the blood. Clinically, patients present with severe metabolic acidosis, failure to thrive, vomiting or other gastrointestinal distress, hypotonia, and degrees of psychomotor retardation. Hepatomegaly, ketonemia, and ketonuria are common, and hypoglycemia, hyperglycinemia, and hyperammonemia may be present. The serum vitamin B_{12} level is normal in these patients. Many children with this

condition die in infancy, usually when the protein catabolism during an acute infection produces an episode of severe metabolic acidosis.

The first cases of the disease were described independently by two European groups in 1967 (18,19). Oberholzer et al. (18) described two unrelated patients with apparently similar symptoms, one who had died at age 2 in 1957 and the other who was born 3 years later. Examination of the stored plasma from the first child and the plasma from the living child revealed high concentrations of plasma ketones and methylmalonic acid. Studies of the living child's urine identified large quantities of methylmalonic acid. This child was found to have a normal response to ingested glucose, but upon ingestion of a 27 gram protein meal, she became hypoglycemic and developed a metabolic acidosis. In addition, the plasma concentrations of ketones and non-esterified fatty acids increased, as did the excretion rate of methylmalonic acid in the urine. A 2 gram oral dose of L-valine produced essentially the same metabolic responses except that urinary methylmalonic acid did not increase; oral loading with sodium propionate gave similar results. An oral load of 30 grams of fat produced minimal changes, and the administration of leucine produced no metabolic changes. Homogenates of this patient's leukocytes produced decreased amounts of succinic, fumaric, and malic acids, but a normal amount of methylmalonic acid, from propionyl CoA and $NaH^{14}CO_3$, as compared to controls.

The Norwegian group of Stokke et al. (19) simultaneously described a child with metabolic acidosis, proteinuria, ketonuria, hepatomegaly, and weight loss who was excreting at least 150 times the normal amount of methylmalonic acid in urine and who was found to have a very high plasma concentration of the acid. Of importance was the fact that two siblings died soon after birth with similar symptoms. Using intravenous ^{14}C- labeled glucose, no radioactivity was found associated with the patient's urinary methylmalonic acid; however, using IV D,L-[^{14}C]valine, a significant amount of the administered radioactivity was recovered as urinary methylmalonic acid after 12 hours. The majority of administed [^{3}H]methylmalonic acid appeared in the urine, almost exclusively in the form of methylmalonic acid. Treatment with cyanocobalamin and adenosylcobalamin (even though the patient had normal vitamin B_{12} level) did not decrease the excretion of methylmalonic acid. On the other hand, a diet low in isoleucine, valine, threonine, and methionine produced a drastic reduction in methylmalonic acid excretion (from 800-1000 mg/24 hr to 100-200 mg/24 hr) within three days and led to weight gain, decreased liver size, and general clinical improvement. Unfortunately, a return to the originally high level and subsequent death occurred in the face of a urinary tract infection and septicemia.

Further characterization of the metabolic abnormalities associated with methylmalonic acidemia was provided by

Lindblad et al. (20) who described a patient with vomiting, weight loss, metabolic acidosis, hypotonia, hepatomegaly, and psychomotor retardation. Depending on the degree of protein intake and the presence or absence of acute infection, the chronic metabolic acidosis was correctable with bicarbonate; however, the urine and plasma invariably had high concentrations of methylmalonic acid. In addition, the child had hyperlactatemia, hyperglycinemia/uria at one time, and ketonuria. At the time of description the child was doing well on protein restriction with a mild chronic metabolic acidosis at the age of 2 3/12 years.

The four cases described above illustrate the clinical and metabolic spectrum of methylmalonic acidemia, and implicate the racemase or mutase step in propionate metabolism as defective. A uniform explanation for the metabolic derangements derived from an apparent block in methylmalonic acid metabolism is difficult to propose with certainty, although some rationale is presented in the legend to figure 2.

Many patients have subsequently been described with methylmalonic acidemia. Elucidation of the biochemical mechanism behind this inborn error of metabolism began with the first description of the disease in this country by Rosenberg and coworkers in 1968 (21). The patient was an 8-month old boy admitted to Yale-New Haven Hospital comatose with decreased serum bicarbonate levels, increased serum and urine ketones, hyperglycinemia/uria, and methylmalonic

Figure 2. Possible biochemical mechanisms for the clinical signs in patients with methylmalonic acidemia. Accumulated methylmalonyl CoA is an inhibitor of pyruvate carboxylase (PC), resulting in decreased gluconeogenesis possibly responsible for the hypoglycemia found in these patients (18). This decreased availability of glucose and glycogen causes release of fatty acids into the bloodstream which are subsequently oxidized to ketone bodies (acetoacetate, beta-hydroxybutyrate and acetone). In addition, the buildup of propionyl CoA precursors can lead to the formation of ketones, contributing to the ketonemia and ketonuria (21). The accumulation of CoA esters in the disease may precipitate the release of CoA from acetyl CoA, producing additional acetone (20). The metabolic acidosis may be caused directly by the methylmalonic acidemia, but this is unclear. Hyperammonemia may be secondary to the inhibition of mitochondrial CPS by accumulated propionyl CoA (22) and hyperglycinemia may be a result of the inhibition of glycine cleavage by propionyl CoA precursors (23).

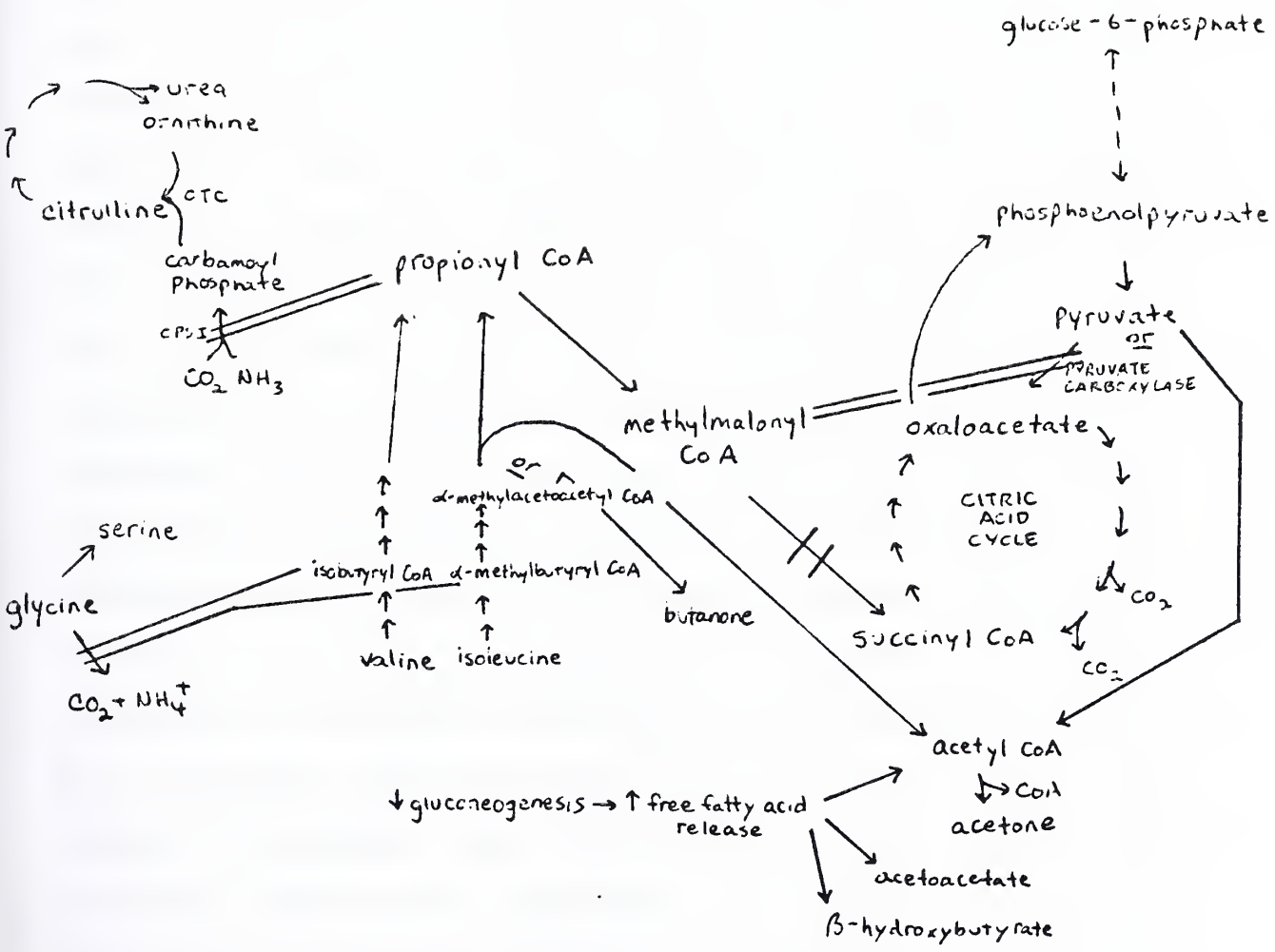


Figure 2. See legend on preceding page.

aciduria. A protein diet high in L-valine or L-isoleucine intensified the boy's signs and symptoms. However, 1 mg of intramuscular vitamin B_{12} per day for five days induced a progressive fall in urine methylmalonic acid excretion from 1100-1250 mg/24 hrs to about 250 mg/24 hrs. Discontinuing the vitamin B_{12} administration caused a rise in the excretion rate in the course of one month to more than 1100 mg/24 hrs. This was the first description of an *in vivo* response to vitamin B_{12} administration in this disease. and thus implicated the mutase rather than the racemase as the site of the deficiency. This vitamin dependency was substantiated *in vitro* by the same group (24) when they suspended leukocytes from this patient in ^{14}C-labeled propionate or succinate and counted the evolved $^{14}CO_2$. The affected child's leukocytes produced negligible $^{14}CO_2$ in the presence of labeled propionate compared to normal leukocytes. During the period when he was receiving vitamin B_{12}, however, his leukocytes were able to oxidize the labeled propionate more effectively. Both normal and affected leukocytes oxidized succinate similarly. Supporting the *in vivo* efficacy of vitamin B_{12}, Lindblad et al. (25) had studied the effect of intramuscular adenosylcobalamin given to their patient one hour before an L-valine tolerance test and had found somewhat less urinary methylmalonic acid excretion over 24 hours compared to the same test without coenzyme administration.

The correction of the clinical and metabolic

manifestations of methylmalonic acidemia with pharmacologic doses of vitamin B_{12} supported the view that either a derangement in the metabolism of the vitamin or a reduced affinity of the mutase apoenzyme for the coenzyme was the underlying biochemical defect in this patient's disease. However, intense *in vivo* and *in vitro* study of subsequent patients revealed more complex information.

In 1969 Morrow et al. (26) measured the adenosylcobalamin content in the livers of four patients with methylmalonic acidemia, and incubated the liver homogenates with 1-[^{14}C]propionate or racemic [^{3}H]methylmalonyl CoA in the presence or absence of added adenosylcobalamin (AdoCbl). In three of these patients, the AdoCbl content in the liver was normal; in one patient however, it was negligible. In the homogenate of this patient's liver, labeled propionate or methylmalonyl CoA was coverted to succinate as well as in controls if cofactor was added, but there was no conversion in the absence of cofactor. In the three patients whose AdoCbl was normal, there was no conversion of either substrate to succinate whether AdoCbl was present in the incubation mixture or absent. In all patients, in the absence of added cofactor there was 3.2 times as much methylmalonic acid accumulated as in controls. Before his death, one of the *in vitro* vitamin B_{12} unresponsive patients had not improved with pharmacologic doses of the vitamin, correlating the *in vitro* evidence with *in vivo* findings.

Since a racemic mixture of methylmalonyl CoA was used in the Morrow study, it supported an abnormal mutase activity rather than a racemase defect. It also established two classes of mutants, one in which there was no appreciable liver AdoCbl and which responded *in vitro* to added cofactor, and another in which there was a normal amount of liver AdoCbl and which did not respond to added cofactor. The relevance of *in vitro* findings to *in vivo* disease was sketchy until the development of methods to study fibroblasts cultured from affected patients. Rosenberg and coworkers (27) studied the fibroblasts from their original *in vivo* vitamin B_{12}-responsive patient and a similar patient. They found that the intact fibroblasts suspended in labeled propionate or methylmalonate could not oxidize either substrate to $^{14}CO_2$ in the presence of 25 pg/ml vitamin B_{12} (oxidation of labeled succinate was normal). When the cells were sonicated after growth in the same concentration of vitamin, there was an insignificant amount of AdoCbl present compared to controls. Only after the vitamin B_{12} concentration was increased 1000 times was the patient's fibroblast AdoCbl concentration normal with near normal oxidation of propionate. In addition, this group showed that for equivalent concentrations of AdoCbl added to extracts of their patient's or control fibroblasts, the enzymatic activity of mutase, measured as the conversion of racemic methylmalonyl CoA to succinyl CoA, was the same. The implication of this finding was that the metabolism of

vitamin B_{12} itself, rather than the relationship between apoenzyme and coenzyme, was deranged in this patient.

While it is attractive to base expected clinical response to administered vitamin B_{12} upon whether fibroblast studies place the patient in the "responsive" or "unresponsive" group, there is much heterogeneity in <u>in vivo</u> resposiveness within the group of responsive fibroblasts (28).

Mahoney et al. (14) further divided the group with abnormal AdoCbl synthesis into two classes on the basis of experiments using cell-free fibroblast extracts which measured the reduction of cob(III)alamin to cob(I)alamin and its subsequent adenosylation. Extracts of controls, of fibroblasts from patients with abnormal AdoCbl synthesis, and of fibroblasts from patients with normal AdoCbl synthesis were incubated with OH-[^{57}Co]Cbl, and the ^{57}Co in the products was measured. The conditions of the assay were such that the cob(III)alamin to cob(I)alamin reduction steps occurred nonenzymatically (29). Interestingly, under these conditions, two subgroups were found within the fibroblasts with abnormal AdoCbl synthesis in intact cells, one with normal AdoCbl synthesizing activity and one with virtually no AdoCbl synthesizing activity. Given the assay conditions, the former group presumably was characterized by a defect proximal to the adenosylation step, perhaps a defective cob(III)alamin reductase, a defective cob(II)alamin reductase or defective delivery of the vitamin

to the site of reduction. This group was named the cbl A class. The biochemical defect in the latter group was presumed to be in the cob(I)alamin adenosyltransferase. This group was named the cbl B class. Incubating extracts from this group with control extracts did not inhibit AdoCbl synthesis by the controls, ruling out the presence of an inhibitor in the cbl B class (14). Subcellular fractionation localized the AdoCbl synthesizing activity to the mitochondria. Fenton and Rosenberg (29) confirmed that cbl B mutant fibroblasts were deficient in cob(I)alamin adenosyltransferase activity, showing that it was undetectable compared to control or cbl A cell lines. In addition, they demonstrated that the adenosyltransferase activity in fibroblasts from the parents of a child with the cbl B defect was about 30% of the activity in control cells.

Characterizing the defects in patients with abnormal AdoCbl synthesis was further complicated by the description of a patient by Mudd et al (30) in 1969 with methylmalonic aciduria unresponsive to pharmacologic doses of cyanocobalamin, in addition to homocystinuria, cystathioninemia, and decreased plasma and urinary methionine. Detailed biochemical studies of this patient demonstrated a decrease in the specific activity of methyltetrahydrofolate methyltransferase with normal plasma levels of N^5-methyltetrahydrofolate. The AdoCbl content of this patient's liver was less than 10% of normal, but the total liver vitamin B_{12} level and plasma vitamin B_{12} level

were normal. More patients were subsequently described with this derangement of both sulfur amino acid and methylmalonic acid metabolism. Mahoney et al (31) cultured fibroblasts from one of these patients in OH-[^{57}Co]Cbl, and found that neither labeled methylcobalamin nor labeled AdoCbl were present, with only 1/3 as much total label accumulated in these fibroblasts as in controls. Fibroblasts from patients with methylmalonic acidemia alone accumulated labeled methylcobalamin normally. and could accumulate as much total label as controls. Patients with the combined sulfur amino acid metabolic defect and methylmalonic acidemia were assigned to the cbl C mutant class. The biochemical defect is surely proximal to the separation of the pathways to the individual coenzymes. Rosenberg and coworkers (32) more clearly defined the cbl C defect. They noted that Cbl C cells lost accumulated cyano[^{57}Co]cobalamin much faster and to a greater extent than control cells, and that this accentuated efflux of accumulated vitamin was probably secondary to the inability of cob(III)alamin reductase to facilitate the reduction of cyanocobalamin.

The heterogeneity of the mutant classes comprising the methylmalonic acidemias was confirmed in genetic complementation studies (33,34). Gravel et al. formed heterokaryons in pairwise combinations from cbl A, cbl B, cbl C, and apoenzyme defect mutants (mut class), using Sendai virus-mediated fusion, and examined [^{14}C]propionate incorporation into trichloroacetic acid precipitable

material. They found that the mutants within each class complemented mutants of all other classes but not of their own class (i.e. [^{14}C] propionate was metabolized properly in all heterokaryons except those formed from two members of the same class) (33). Willard, Mellman and Rosenberg (34) used polyethylene glycol-induced heterokaryons and confirmed the heterogeneity of the cbl mutant classes. In addition, they found two cell lines from siblings with the clinical hallmarks of the cbl C class which complemented not only other mutant classes but also all other cbl C lines. These two lines did not complement each another, and hence were assigned to a cbl D mutant class. They also examined ^{14}C incorporation into trichloroacetic acid precipitable material from N^5-[^{14}C]methyltetrahydrofolate and found cbl C and cbl D mutants complementary to one another but not to themselves. Such CxD heterokaryons accumulated 30-40% more labeled cobalamin and synthesized five times more AdoCbl than CxC, DxD or C or D alone. Hence, complementation analysis confirmed the existence of five mutant classes which result in deficiencies in the activity of methylmalonyl CoA mutase: cbl A, cbl B, cbl C, cbl D, and mut, the class with normal AdoCbl synthesis, presumably a mutase apoenzyme mutant.

During the last five years much work has gone into characterizing more accurately the group of mutants which synthesize coenzyme normally and, therefore, are presumably apoenzyme mutants. In 1977, Willard and Rosenberg (35)

studied the growth of such *mut* mutant fibroblasts in cobalamin- supplemented media and examined the reaction kinetics of methylmalonyl CoA mutase in those fibroblast extracts. They found that two of the five mutants studied, though unable to fix [1-^{14}C]propionate in basal medium, could fix propionate when high doses of OH-Cbl (10-1000 pg/ml) were added to the growth medium. Both of these mutants had normal AdoCbl synthesis and were verified to be apoenzyme mutants by complementation analysis. When the mutase activity in extracts of these cells was studied using various concentrations of added AdoCbl, these two mutant lines had significantly higher K_m's for AdoCbl (2.8×10^{-4} M and 1.7×10^{-5} M) compared to control mutase ($6\text{-}7 \times 10^{-8}$ M) and had V_{max} values 20% and 5% of control values. The other three mutant lines examined had undetectable mutase activity for all AdoCbl concentrations studied. Hence, two subgroups within the *mut* complementation class exist, one with a reduced affinity for coenzyme demonstrated by an increased K_m for AdoCbl (*mut*$^-$ mutant) and the other with no detectable mutase activity at any AdoCbl concentration (*mut*^{0} mutant). The existence of the *mut*$^-$ group was supported by Morrow et al. (36), who found a much increased K_m for AdoCbl but a normal K_m for D,L-methylmalonyl CoA when studying the mutase activity in fibroblast extracts from a patient with normal synthesis of AdoCbl who lowered her urinary excretion of methylmalonic acid in response to intramuscular vitamin B_{12}.

Using 23 apoenzyme mutant fibroblast lines verified by

complementation analysis and normal cobalamin metabolism studies, Willard and Rosenberg (37) separated the mutants into 16 *mut*^{0} lines and 7 *mut*$^{-}$ lines based on their refractoriness or resposiveness to OH-Cbl supplementation and on the basis of kinetic studies using fibroblast extracts. The reaction kinetics of mutase with respect to AdoCbl concentration in the 7 *mut*$^{-}$ lines were such that 6 of the *mut*$^{-}$ lines conformed to simple Michaelis- Menten kinetics with a higher K_m for AdoCbl compared to control mutase. One of the *mut*$^{-}$ lines followed complex kinetics suggesting that it was derived from a compound heterozygote for mutase deficiency. Further studies on two of the *mut*$^{-}$ lines showed that their K_m for D,L-methylmalonyl CoA was normal. When excess AdoCbl was added to extracts from these two *mut*$^{-}$ lines, their mutase activity after incubation at 45°C was significantly less than at 37°C. Control mutase was not inactivated by this temperature in the presence of excess AdoCbl. When the turnover time of mutase was studied in the presence of an inhibitor of protein synthesis, they found that the half-life ($t_{1/2}$) for mutase in a *mut*$^{-}$ mutant was 3-4 days, compared to the control mutase $t_{1/2}$ of 7-14 days. Hence, the mutase protein from the group of mutants with decreased affinity for coenzyme appears to be less stable than control mutase.

Willard and Rosenberg (37) also examined fibroblasts from the presumptively heterozygous parents of apoenzyme mutant children. Extracts from cell lines grown in basal

medium demonstrated mutase activity within the control range. In the presence of normally saturating concentrations of AdoCbl, however, these parents demonstrated mutase activity well below that of controls with added AdoCbl. When the same experiments were done after growth in hydroxocobalamin-supplemented medium, the parents' mutase activity was only 1/2 that of control mutase. These data demonstrate some reduced activity of the parents' mutase enzyme. When the kinetics of mutase in extracts of 8 heterozygote lines were studied, 5 of them (4 parents of *mut*$^-$ mutants and 1 parent of a *mut*^{0} mutant) displayed more activity at 1 mM AdoCbl than at 6 uM, a concentration which is saturating for normal mutase. In the parents of the *mut*$^-$ mutants, complex kinetics with respect to AdoCbl saturation were found, with two apparent K_m's, one similar to the control K_m and another K_m corresponding to the affected child's K_m, providing excellent evidence for *mut*$^-$ heterozygosity in both parents. Fibroblast extracts from the parents of a *mut*$^-$ mutant child with 23% of original mutase activity after a 45°C incubation showed 84% and 75% of their original mutase activities after a 45°C incubation. Detailed kinetic studies of the parents of a *mut*^{0} child showed that one parent possessed normal mutase kinetics and the other parent had a Michaelis-Menten plot similar to that of a *mut*$^-$ heterozygote. This suggests that the child had a *mut*^{0}/*mut*$^-$ genotype, the product of *mut*^{0}/*mut*$^+$ and *mut*$^-$/*mut*$^+$ parents.

Kolhouse et al. (38) developed a radioimmunoassay to quantitate material cross-reacting immunochemically with human mutase. They found that fibroblast extracts from controls and cbl A,B,C and D mutants contained similar amounts of cross-reacting material (CRM) per mg of cell protein. The mut^- lines tested contained 20-100% of control CRM, as one would expect from an enzyme which has measurable mutase activity. Interestingly, of the 21 mut^0 lines tested, CRM was undetectable in 12 lines and varied from 3% to 40% of control CRM in the other 9. The mut^0 line with the highest CRM was studied and its CRM was found to differ very little in its anion exchange chromatography elution profile (i.e. charge and size) compared to the CRM from control cells. Therefore, within the mut^0 group there exist two subgroups, one which contains material cross-reacting immunochemically with human mutase, and one which does not contain such material. Explanations for the CRM^- group include a drastically altered enzyme bearing no relation to normal mutase, a non-existent enzyme perhaps secondary to deletion mutations, mutations related to regulation of DNA or RNA processing, or defects related to its incorporation into the mitochondrion, which will be discussed in greater detail later. The CRM^+ subgroup has fewer possible explanations; a single base mutation may eliminate the enzyme's biochemical activity yet not eliminate its presence from the cell, or a defect related to mitochondrial incorporation may again be suggested.

In summary, five complementation classes have been defined to describe the biochemical defects manifesting themselves as methylmalonic acidemia. The cbl A,B,C and D classes each are defective in some aspect of AdoCbl synthesis (see figure 3) which are correctable *in vitro* and in most cases *in vivo* with pharmacologic doses of vitamin B_{12}. The *mut* complementation class is characterized by normal AdoCbl synthesis and contains two groups within it, the *mut*$^-$ group in which the affinity of mutase for cofactor is reduced, and the *mut*^{0} group, in which mutase activity is nonexistent, and is comprised of CRM^+ and CRM^- subgroups. The study of the *mut* complementation class has led to interesting questions regarding the synthesis and subsequent handling of methylmalonyl CoA mutase and other enzymes which are made in the cytosolic compartment but act within the mitochondrion. That methylmalonyl CoA mutase is a cytoplasmically synthesized enzyme coded for by nuclear DNA is demonstrated by the autosomal recessive inheritance of the methylmalonic acidemias.

Transport Characteristics of Mitochondrial Proteins Synthesized in the Cytoplasm

Although the mitochondrion has the capability to synthesize proteins using its own DNA, RNA and ribosomes, only a small fraction of the proteins which function within the mitochondrion are actually formed there. In fact, 90% of the mitochondrial proteins are translated on cytoplasmic ribosomes from mRNA transcribed from nuclear DNA (39,40).

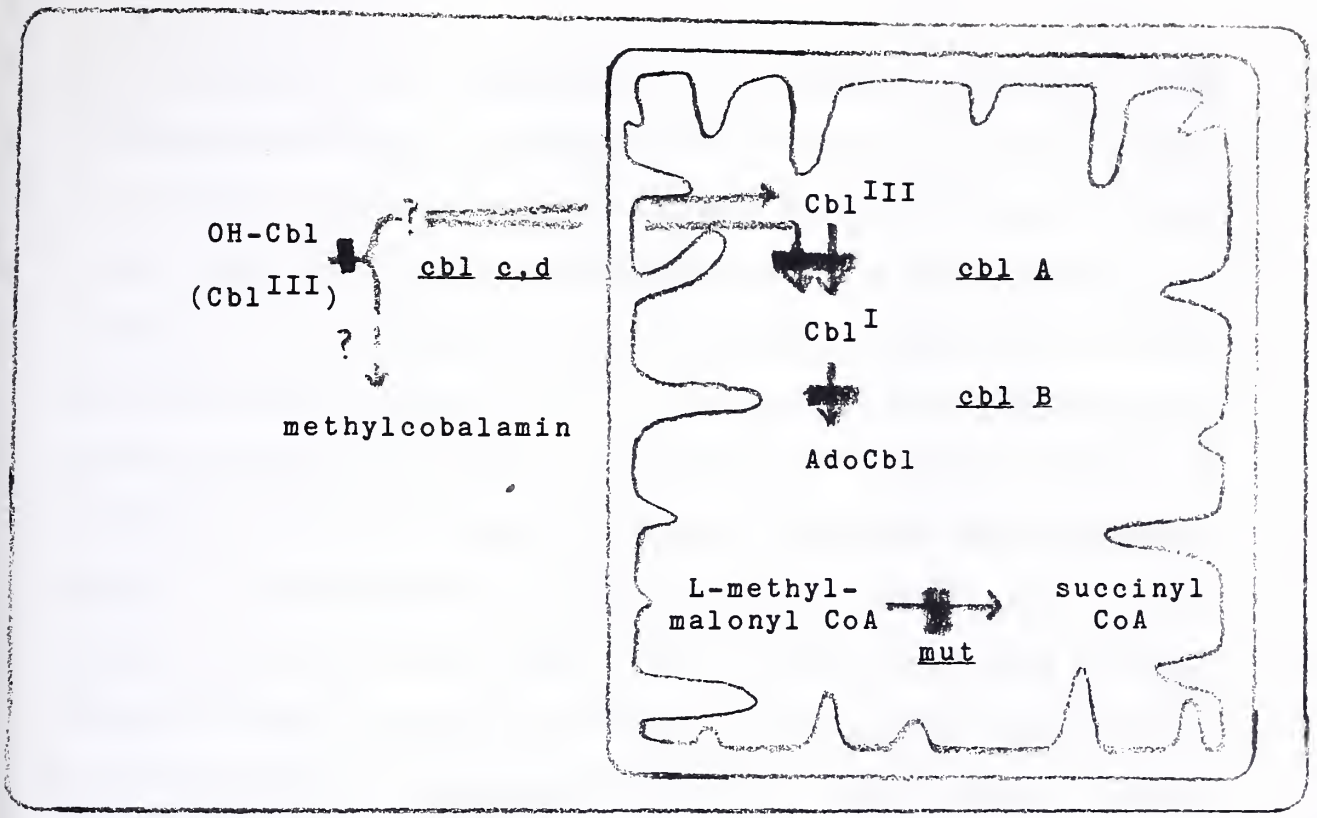


Figure 3. Presumptive blocks in the sequence of cobalamin metabolism and conversion of L-methylmalonyl CoA to succinyl CoA (see text).

The mitochondrion consists of an outer membrane, the intermembrane space, an inner membrane and the matrix space. This means that not only do these proteins have to move within the cell to the mitochondrion, but also, depending on where in the mitochondrion they function, they have to cross an additional membrane or two. Intracellular compartment-to-compartment transfer is not a novel concept; proteins synthesized in the cytosol which are destined for secretion appear to be made on polyribosomes attached to the endoplasmic reticulum (ER). The proteins contain an NH_2-terminal signal sequence of amino acids which guides them into the ER for subsequent export. This leader signal sequence is removed as the protein is still being translated and is moving across the ER membrane, a process termed "vectorial translation" (41,42). An important difference between this system and the movement of proteins into the mitochondrion is that experiments have not been able to establish that cytoplasmic polyribosomes which are closely associated with the mitochondrion necessarily translate mRNA for mitochondrial proteins (43). In fact, it has been shown that the mitochondrial enzyme ornithine transcarbamylase (OTCase) is synthesized exclusively on free polysomes (44). This direct evidence, along with other indirect evidence (39), implies that these proteins are likely synthesized on free polyribosomes. Certain similarities between the two systems do exist however, and the properties of the transport and processing of cytoplasmically synthesized

mitochondrial proteins have been studied with growing interest over the last 5 years.

Before most of the work on mitochondrial proteins began, Chua and Schmidt (45), among others, studied the transport of the cytoplasmically synthesized small subunit of ribulose-1,5-bisphosphate carboxylase into chloroplasts. Using a wheat germ cell-free translation system programmed withmRNA isolated from pea and spinach, they were able to immunoprecipitate with ribulose-1,5-bisphosphate carboxylase antiserum a polypeptide proving to be about 4000 daltons larger than the enzyme immunoprecipitated from whole pea or spinach cells grown continuously in labeled methionine. After the addition of isolated pea or spinach chloroplasts to the in vitro translation mixture, immunoprecipitation yielded only the mature sized enzyme, which proved to be identical to ribulose-1,5-bisphosphate carboxylase synthesized by whole cells.

Similar experiments were subsequently performed using antisera made against mitochondrial proteins synthesized in the cytoplasm. Macchecchini et al. (46) used a rabbit reticulocyte lysate translation system containing [^{35}S]methionine to which yeast mRNA was added to study synthesis of the three largest subunits of the yeast F_1-ATPase. This enzyme is located on the matrix side of the mitochondrial inner membrane and is composed of five nonidentical subunits. Using antibodies against each of the cytoplasmically made subunits, three bands corresponding to

molecular weights of 64,000 (alpha), 56,000 (beta) and 40,000 (gamma) daltons could be visualized on the autoradiograph of the sodium dodecyl sulfate-polyacrylamide gel. Compared to the immunoprecipitated proteins from yeast cells grown continuously in $^{35}SO_4^{2-}$, the *in vitro* translated products were larger by 6000, 2000 and 6000 daltons respectively. In another experiment, these workers pulsed yeast spheroplasts with [^{35}S]methionine for only five minutes, and found that the immunoprecipitate of the lysed cells contained both the larger forms of the subunits as translated *in vitro* and the mature forms of the subunits as found after continuous labeling *in vivo*. When a 45 minute chase with unlabeled methionine followed the 5 minute pulse, the larger forms of the subunits could no longer be found. To show that the larger precursor forms were processed by mitochondria into the smaller mature forms, mitochondria were added to the *in vitro* translation system after protein synthesis had been stopped. After centifugation to separate these mitochondria from the rest of the mixture, immunoprecipitation demonstrated that the mitochondrial pellet contained both precursor and mature forms of the subunits whereas the mitochondria-free supernatant contained only the precursor forms. The addition of proteases before centrifugation eliminated all radioactive product from the supernatant fraction, but the mitochondrial pellet lost only the precursor forms; the mature subunits were protected from protease activity and could still be immunoprecipitated.

This evidence implies that the precursor forms originally found with the pellet were either nonspecifically associated with the mitochondria, perhaps adhering to their outer surfaces, or not yet transported into the inner membrane (outer membranes are not intact after this treatment and leakage from the intermembrane space is possible). This group also showed that mitochondria do not import mature subunits, a result which was duplicated by Gasser et al. (47) using a mature subunit of yeast cytochrome c oxidase. Macchecchini et al. and others demonstrated by proteolytic fingerprinting that the alpha, beta and gamma precursor forms of yeast F_1-ATPase are structurally similar to the mature forms (46,48).

That these F_1-ATPase subunits are synthesized individually in the cytoplasm and not as a polyprotein which could move into the mitochondrion as a unit was proven using unlabeled L-methionine and N-formyl[^{35}S]methionine-tRNA$_f$ in the <u>in vitro</u> translation mixture (48,49). With this system, only the NH_2-terminal methionine would be radioactively labeled and all other methionines would be unlabeled. Indeed, the autoradiograph revealed distinct radioactive bands corresponding to the individual F_1-ATPase alpha, beta and gamma subunit precursor forms (48,49). With the addition of mitochondria to the translation mixture after terminating protein synthesis, these bands no longer appeared, implying that in the process of becoming mature subunits, the mitochondrion cleaves an NH_2-terminal sequence

of amino acids from the precursor proteins (48). Similarly, the four cytoplasmically synthesized subunits of yeast mitochondrial cytochrome c oxidase were demonstrated to be made individually using N-formyl-[^{35}S]methionine $tRNA_f$ as the only radioactive species in the translation mixture (49).

Other mitochondrial proteins synthesized in the cytoplasm have been studied in a fashion similar to that for the F_1-ATPase. For example, the cytochrome bc_1 complex is composed of six cytoplasmically synthesized subunits and one subunit made on mitochondrial ribosomes and spans the mitochondrial inner membrane. Using yeast mRNA and a reticulocyte lysate translation system, subunit V was found to move more slowly on a sodium dodecyl sulfate-polyacrylamide gel than the subunit immunoprecipitated from continuously labeled yeast spheroplasts. Short pulse labeled spheroplasts made both forms of the subunit, and subsequent chase in the absence of radioactivity left only the 2000 dalton smaller mature form. The presence of protease inhibitors blocked processing and left only the larger form (50).

An intermembrane space enzyme, cytochrome c peroxidase, was immunoprecipitated from an *in vitro*, yeast mRNA programmed translation system and from continuously labeled yeast spheroplasts, short pulsed yeast spheroplasts, and pulse-chased yeast spheroplasts, with results similar to those described above (51). Like the F_1-ATPase, when

mitochondria were added to the products of the translation and then recovered by centrifugation, the pellet contained both precursor and mature forms of cytochrome c peroxidase if no proteases were present, but contained only the mature form in the presence of proteases. The supernatant produced no radioactive bands in the presence of proteases, and contained both mature and precursor forms in their absence (51). An explanation for the presence of mature enzyme in the supernatant may relate to a disturbance of the outer membrane during isolation of mitochondria; if the membrane is not intact, any enzyme which is normally sequestered to the intermembrane space may escape into the supernatant fraction.

Cytochrome c oxidase is the last member of the electron transport chain, and is composed of subunits I-III which are made on mitochondrial ribosomes and subunits IV-VII which are made on cytoplasmic ribosomes. Using a cell-free wheat germ translation system programmed with rat liver mRNA, subunit IV of this inner membrane enzyme was shown to be synthesized as a precursor 3000 daltons larger than the mature form; tryptic fingerprinting confirmed close similarities in their amino acid structures (52). Using yeastmRNA in a reticulocyte lysate translation system and comparing the immunoprecipitated products to those from continuously labeled yeast cells, subunits IV, V, and VI were found to be 1500-3000 daltons larger in the <u>in vitro</u> system, whereas subunit VII did not appear to be synthesized

in a larger precursor form (49). Although there seems to be precedent for uncleaved signal sequences (51,49), the absence of a precursor form of cytoplasmically synthesized mitochondrial proteins appears to be the exception rather than the rule.

Nelson and Schatz (53) studied the energy requirements for the transport and processing of cytoplasmically synthesized precursors to inner membrane proteins in yeast spheroplasts. Because ATP can diffuse freely through the outer mitochondrial membrane but not through the inner membrane, mitochondrial matrix ATP must be derived from either oxidative phosphorylation of ADP to ATP by the respiration-linked F_1-ATPase which protrudes into the matrix space orby importing ATP from the cytoplasm via the inner membrane adenine nucleotide transporter. In their experiments, they selectively blocked the respiratory chain (using KCN, antimycin A, or a mutation known as *rho*$^-$) and/or the ATP import system (using bongkrekic acid or the *op*$_1$ mutation") in labeled yeast spheroplasts. Neither KCN, antimycin A, nor bongkrekic acid could inhibit the *in vivo* processing of the alpha, beta, or gamma subunits of the F_1-ATPase, a subunit of the cytochrome bc_1 complex, or cytochrome c_1 in wild-type spheroplasts. However, KCN added to cells with the *op*$_1$ mutation, antimycin A and bongkrekic acid added together to wild-type cells, and bongkrekic acid added to cells with the *rho*$^-$ mutation were all able to block processing of the precursors to the mature forms. In

addition, carbonyl cyanide m-chlorophenylhydrozone (CCCP), an uncoupler of oxidative phosphorylation, inhibited processing of each protein. From these studies it appears that matrix ATP is necessary for the translocation and/or processing of these inner membrane proteins, but the relationship of processing to translocation has not yet been clearly defined.

Studies using mammalian cells are coming closer to clarifying the processing system. Shore et al. (54) showed that carbamyl phosphate synthetase I, a liver mitochondrial matrix enzyme which catalyzes the first step of the urea cycle in ureotelic animals, is made as a 5500 dalton-larger precursor by a rabbit reticulocyte lysate translation system programmed with rat liver polysomal mRNA. Peptide mapping showed this precursor to be structurally very similar to the mature form immunoprecipitated from isolated rat liver mitochondria. Similarly, Conboy et al. showed that ornithine transcarbamylase, a matrix enzyme catalyzing the second step of the urea cycle and composed of three identical 36,000-39,000 dalton subunits, is synthesized in the cytoplasm as a ~43,000 dalton precursor (55). Mori et al. (56) found that, when a translation mixture was incubated with rat liver mitochondria, a polypeptide intermediate in size between pre-OTCase and mature OTCase was formed in addition to the mature OTCase. Processing of the precursor form to either smaller form was completely inhibited when OTCase antiserum was added to the incubation

mixture. Conboy and Rosenberg (57) also reported a minor band representing an intermediate form of OTCase in the mitochondrial pellet after centrifugation of a translation mixture to which mitochondria were added, along with a minor band representing pre-OTCase and a major band in the position of mature OTCase. After chymotrypsin treatment, however, the pellet contained only the mature form. Mori and coworkers claimed to localize the processing activity converting the pre-OTCase to intermediate-OTCase to the matrix space using submitochondrial fractions (56).

Conboy et al. (58) also subfractionated rat liver mitochondria in an attempt to localize the processing protease activity responible for the conversion of *in vitro* synthesized pre-OTCase to intermediate-OTCase and mature OTCase. They found the activity producing intermediate sized OTCase divided equally among the inner membrane and matrix fractions. Examining the conversion to mature OTCase produced more conclusive results, however. No subfraction could process the precursor to mature OTCase in the absence of added Zn^{2+}, although processing to intermediate-OTCase was unaffected by the absence of the ion. All of the activity responsible for the appearance of mature OTCase could be localized to the mitoplast subfraction (inner membrane + matrix); further separation localized >90% of the processing activity to the matrix and <10% to the inner membrane, the latter consistent with matrix contamination according to marker enzyme studies. The appearance of

mature OTCase was found to be Zn^{2+} concentration-dependent up to 0.1 mM and was inhibited by the divalent cation chelator, 1,10-phenanthroline. Among other cations tested, Co^{2+} also supported processing, as did Ca^{2+} and Mn^{2+} to a lesser extent. Hence, processing to the mature OTCase appeared to take place in the matrix space by the action of a Zn^{2+}-dependent protease whose Zn^{2+} requirement (0.1 mM) corresponds to physiologic mitochondrial matrix Zn^{2+} concentrations. Gasser et al. (46) supported the theory of a divalent cation-dependent protease localized to the matrix by demonstrating that processing of the *in vitro* translated pre-beta subunit of the yeast F_1-ATPase by mitochondrial extracts could be inhibited by GTP, which acts as a divalent cation chelator in the absence of saturating Mg^{2+} concentrations. In contrast, intact mitochondria processed the subunit normally in the presence of GTP; because GTP cannot permeate the mitochondrial inner membrane, such processing must therefore have occurred in the matrix space or inner membrane.

The energy dependence of the translocation and processing of pre-OTCase was studied by Kolansky et al. (59) using rat liver mitochondria and the products of a rat liver mRNA programmed rabbit reticulocyte lysate translation system. While inhibitors of the electron transport chain reduced the formation of mature enzyme, inhibitors of the F_1-ATPase and the adenine nucleotide translocation system had no effect on the processing to mature OTCase.

Uncouplers of oxidative phosphorylation, such as 2,4-dinitrophenol (DNP), completely inhibited the appearance of mature OTCase in a concentration dependent fashion, but had no effect on the appearance of the intermediate form. If the matrix subfraction was used in place of intact mitochondria, processing was complete in both the absence or presence of DNP. If the whole mitoplast subfraction was used, DNP inhibited the appearance of mature OTCase but not the intermediate form. When intact mitochondria were recovered after processing, the mature enzyme was recovered in the pellet and the intermediate form in both the supernatant and pellet in the absence of DNP. In the presence of DNP, only the intermediate form could be recovered, and was found in the extramitochondrial supernatant fraction.

This evidence supports the concept that the precursor form is cleaved to the intermediate form before translocation across the inner membrane occurs, and that this translocation, rather than the processing itself, requires energy. The observation that no precursor form is found strongly associated with the mitochondrial fraction argues against a time lag between translocation and processing, and supports the concept of "vectorial processing" (i.e. matrix processing occurring as the protein is being translocated) analogous to the "vectorial translation" of proteins destined for secretion into the rough endoplasmic reticulum. To further characterize the

energy source, Kolansky et al. (59) chromatographed the translation mixture to remove ATP and other small molecules; the addition of mitochondria to this depleted mixture did not result in processing of the pre-OTCase to mature OTCase. Only after adding back ADP, ATP, or untreated lysate could processing be reestablished. Gasser et al. (46) showed that when small molecules were filtered out of a reticulocyte lysate translation system programmed with yeast mRNA, the pre-beta subunit of the F_1-ATPase also could not be converted to its mature form by added intact mitochondria. Addition of ATP to the incubation mixture restored processing. Furthermore, addition of lysed mitochondria to the ATP-depleted translation mixture resulted in normal processing, implying that translocation, not processing, is energy dependent in this case as well.

It seems reasonable that a matrix enzyme such as OTCase should depend upon a matrix protease for processing and an energized inner membrane for translocation. However, Reid et al. (60) showed that two intermembrane space proteins, cytochrome b_2 and cytochrome c peroxidase, also rely on an energized inner membrane and a 1,10-phenanthroline-sensitive protease for processing. They pulse-labeled yeast spheroplasts with [^{35}S]methionine in the presence of CCCP and found only precursor forms of the proteins. After removal of the CCCP, pre-cytochrome b_2 and pre-cytochrome c peroxidase proceeded to their mature forms, cytochrome c peroxidase directly, and cytochrome b_2 via an intermediate

polypeptide like that described for OTCase. Processing of both of these precursors also was blocked by 1,10-phenanthroline. It was shown using *in vitro* translation that the intermediate form of yeast cytochrome b_2 was produced from its precursor by an activity localized to the matrix of subfractionated yeast mitochondria which was sensitive to 1,10-phenanthroline (61). Daum et al. showed that mitochondria could accumulate the intermediate when exposed to *in vitro* translated precursor at low temperature; they took advantage of this finding to demonstrate that the second processing step, to mature cytochrome b_2, is not affected by the presence of 1,10-phenanthroline or uncouplers of oxidative phosphorylation (61). With pulse-labeled yeast spheroplasts, Reid et al. localized the intermediate to the inner membrane subfraction after processing (60). Though it is not clear that this matrix-localized, 1,10-phenanthroline-sensitive protease is the same as that described for the processing of rat OTCase, in the cases of these intermembrane space proteins, it is the translocation and processing of the intermediate form, rather than the mature form, which requires an energized inner membrane and a matrix protease.

The processing of cytochrome c_1, an inner membrane protein which is part of the cytochrome bc_1 complex, also appears to occur via an intermediate form whose appearance depends upon a 1,10-phenanthroline-sensitive matrix protease (62). Ohashi et al. took advantage of the observation that

processing to mature cytochrome c_1 requires heme. and used a yeast mutant lacking heme to accumulate the intermediate form, 4000 daltons larger than mature cytochrome c_1 and 2000 daltons smaller than *in vitro* translated precursor (62). That this intermediate form precedes the mature form was proven by chasing the pulsed mutant cells in the presence of added heme precursor and observing the conversion of intermediate to mature cytochrome c_1.

Finally, the kinetics of translocation and processing have been studied with pulse-chase experiments performed in the absence or presence of an inhibitor of the mitochondrial processing system (63,64,65). Using rat liver explants, Raymond and Shore (63) showed that [^{35}S]methionine labeled pre-carbamyl phosphate synthetase (pre-CPS) disappeared within a 4-minute chase period, with an apparent $t_{1/2}$ for conversion of pre-CPS to mature CPS of about 2 minutes. Raymond and Shore then blocked processing with p-aminobenzamidine, a protease inhibitor, and found that with time, less pre-CPS could be found within the extramitochondrial fraction of the explant homogenates but no mature CPS appeared in the mitochondrial fraction. The $t_{1/2}$ for total disappearance of the pre-CPS was about 2-3 minutes. Mori et al. (64) pulsed rat hepatocytes with [^{35}S]methionine and then chased for various times with unlabeled methionine and demonstrated that the precursor form of OTCase disappeared from the cells' cytoplasmic fraction within 10 minutes of chase, as the radioactivity in

the particulate fraction increased. The $t_{1/2}$ for pre-OTCase conversion to OTCase in this system was about 2 minutes, similar to that for CPS. The precursor form of another cytoplasmically synthesized mitochondrial protein, aspartate aminotransferase, could be chased into the mature mitochondrial form with a $t_{1/2}$ of about 30-60 seconds in chicken embryo fibroblasts (65). Including CCCP in the pulse-chase incubation mixture caused all of the labeled precursor to disappear within 15 minutes with a $t_{1/2}$ of about 5 minutes, without any incorporation of label into the mature form of the enzyme. These kinetic studies all agree that the half-time for translocation and processing is on the order of a few minutes, and that when either is blocked, the precursor forms of the proteins destined for the mitochondrion appear to be degraded within minutes.

To summarize the evidence accumulated over the recent years from both yeast and mammalian studies, it appears that most mitochondrial proteins which are synthesized on cytoplasmic ribosomes are made as larger precursor forms which are converted post-translationally into the mature proteins. Not all such proteins are made as larger forms, however; whether their size or tertiary structure obviates the need for the additional amino acid sequence(s) remains to be shown. That cytoplasmic ribosomes which translate precursor proteins do not appear to be necessarily associated with mitochondria is indirectly supported by the observations that 1) a lag time exists between the

appearance of cytoplasmic precursor and the appearance of mitochondrial enzyme, 2) precursor has never been found to be intimately attached to the mitochondrial fraction of cells *in vivo*, 3) precursor is easily detected in the extramitochondrial compartment of fractionated cells, 4) analysis of mRNA on polysomes associated with mitochondria does not demonstrate that they code preferentially for mitochondrial proteins, and 5) OTCase is synthesized exclusively on free polyribosomes. The NH_2-terminal "signal sequence" of amino acids characterizing precursor forms is cleaved within the mitochondrial compartment. It is attractive to postulate that this sequence is recognized by mitochondrial outer membrane receptors which make it possible for these proteins to enter the mitochondrion. Even if free protein recognizes its mitochondrial destination via signal sequence-membrane receptor interactions, the process by which the protein is translocated across the mitochondrial outer membrane is unclear. Since ATP formed from substrate level phosphorylation in the cytoplasm is freely permeable across the outer membrane, an ATP-dependent translocation theory is difficult to test in intact cells. Whatever the mechanism for translocation across the outer membrane, it appears that whether proteins are destined for the intermembrane space, the inner membrane, or the matrix space, translocation across the inner membrane occurs. This translocation is energy dependent because it can be blocked by inhibiting ATP

formation at the inner membrane-matrix interface and inhibiting ATP access across the inner membrane. Processing, however, does not seem to be ATP-dependent. For pre-OTCase, a matrix enzyme precursor, the processing activity can be localized to the mitoplast fraction, implying that a pre-mature form of OTCase still exists within the intermembrane space. Whether the "signal sequence" or another portion of the protein recognizes the inner membrane remains unclear. Perhaps cleavage to the intermediate form of OTCase prohibits its exit into the cytoplasm and is necessary for its complete translocation into the matrix space.

It appears that two distinct proteases are responsible for the processing of pre-OTCase to mature OTCase, a Zn^{2+}-independent protease whose activity is localized to the mitoplast fraction and which cleaves pre-OTCase to intermediate-OTCase, and a Zn^{2+}-dependent protease which processes the protein to mature OTCase in the matrix space. However, evidence has accumulated that intermembrane space enzymes (cytochrome b_2, cytochrome c peroxidase) and inner membrane enzymes (cytochrome c_1, F_1-ATPase subunits) also require a matrix-localized divalent cation-dependent protease. In the cases of cytochrome b_2 and cytochrome c_1, in which intermediate forms have also been identified, processing the precursor to the intermediate form requires this protease and an energized inner membrane, whereas processing to the mature form does not. Whether processing

occurs concomitantly with translocation across the inner membrane has yet to be directly shown, but it appears that "vectorial processing" is a valid concept, especially because there is no evidence that proteins which require a matrix protease for processing, yet are localized in a mitochondrial subcompartment other than the matrix, can at some point be found in the matrix.

For the proteins so far studied, if translocation or processing is blocked, the precursor form disappears from the cell within minutes, presumably degraded. This degradation has not been examined closely, although the concept is certainly not new. Limited studies suggest that abnormal proteins are degraded rapidly by intracellular proteases; the details of the recognition of such abnormal proteins are not well worked out, but it may be based on their size, charge, tertiary structure or interactions with other proteins (e.g. ubiquitin) and with each other (66).

Thus, mitochondrial protein biogenesis is a complicated matter without enough evidence accumulated so far to construct a scheme generally applicable to cytoplasmically synthesized mitochondrial proteins. In the studies which follow, the *in situ* characteristics of Buffalo rat liver methylmalonyl CoA mutase are described with special attention to its cytoplasmic form, turnover rate and mitochondrial processing.

MATERIALS AND METHODS

Materials

Buffalo rat liver cells (clone BRL-T) were supplied by Dr. Elizabeth Neufeld, NIH. Dulbecco's modified Eagle's minimal essential medium, Eagle's minimal essential medium, kanamycin, and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Grand Island Biological Company. Fetal bovine serum was from Sterile Systems, Inc. L-[4,5-^{3}H]leucine (47 Ci/mmol, 1 mCi/ml) was purchased from Amersham. Formalin-fixed Staphylococcus aureus cells were supplied by Bethesda Research Laboratories. 2,4-dinitrophenol was purchased from Sigma. Unlabeled L-leucine was from Schwarz-Mann. Coomassie brilliant blue R-250 was purchased from Bio-Rad Laboratories. Other reagents used were from usual commercial sources.

Culturing Buffalo rat liver (BRL) cells

BRL cells were cultured in Falcon 75 cm^2 plastic tissue culture flasks containing 25 ml of Dulbecco's modified Eagle's minimal essential medium supplemented with0.05% (v/v) kanamycin (10,000 mcg/ml) and 5% (v/v) fetal bovine serum (referred to hereafter as Dulbecco's modified medium). The cells were grown in 5% CO_2-95% air at 37°C. Upon reaching confluence, the cells were transferred into Costar 100 mm dishes. Cell transfer was performed under sterile conditions and consisted of first removing the growth media

and rinsing the flasks once with about 10 ml of phosphate-buffered saline (0.14M NaCl, 3mM KCl, 10mM Na_2HPO_4, pH 7.0). Then, 1.5 ml of 0.25% trypsin-EDTA (0.2 g/l) was added to the flasks to cover the cells and the flasks were incubated at 37°C for 1-2 minutes until the cells lifted off the flask as confirmed by microscopic evaluation. Approximately 7-10 ml of the Dulbecco's modified medium was pipetted into the flasks in such a way as to ensure dislodging of all the cells still adhering to the bottom of the flask and to each other. Aliquots of the flask contents were pipetted into the dishes (or new flasks) and 10 ml of Dulbecco's modified medium were added to each dish (or 25 ml to each new flask). Cells were usually divided into 2-6 new flasks or dishes per original flask. Confluence could be achieved in 1-3 days, depending upon the extent of the dilution. Upon confluence, the dishes of cells were used in experiments.

Labeling and harvesting of BRL cells and immunoprecipitation of mutase

For each experimental time point three dishes of cells were used. The culture medium was removed from each dish, and they were rinsed with about 5 ml phosphate-buffered saline. The cells were then incubated for 60 minutes at 37°C in 8 ml of Eagle's minimal essential medium without leucine and with 10% dialysed fetal bovine serum. The medium from each dish then was removed and replaced with 4 ml Eagle's leucine-deficient media and 0.2 ml L-

[^{3}H]leucine (47 Ci/mmol). The cells were incubated at 37°C for 2 hours. All the medium was then removed, the cells were rinsed with 5 ml cold phosphate-buffered saline (~4°C), and the cells were immediately lysed while still attached to the dish by adding 1 ml NETS-leu buffer (0.15M NaCl, 0.01M EDTA, 0.5% Triton X-100, 0.25% sodium dodecyl sulfate (SDS), 1% L-leucine) per dish. The lysates from each of the three plates were combined and centrifuged for 20 minutes at 36,750 x g in a Beckman L-75B ultracentrifuge to remove particulate matter. The supernatant was collected (3-4 ml) and 8 µl of mutase antiserum prepared previously against human liver mutase (5) was added to it. The mixture was left at room temperature for 30 minutes and then refrigerated at 4°C overnight. In one experiment, competition for antiserum between purified unlabeled mutase from human liver (5) and the putative mutase protein in the labeled cell extract was tested by dividing the supernatant equally and adding only antiserum to one aliquot, and both 0.03 mg of purified mutase and 8 µl of antiserum to the other.

Recovery of immunoprecipitated mutase

To each sample immunoprecipitated overnight, 90 µl of Staphylococcal aureus cell suspension (10% w/v) were added and the mixture incubated at room temperature for 10 minutes. To separate the S. aureus - antibody - mutase complexes, the aliquots were centrifuged for 10 minutes at

6975 x g in a Sorvall RC-3 general purpose centrifuge. The precipitate from each aliquot was resuspended in 0.5 ml RIPA buffer (10 mM Tris-Cl (pH 7.2), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% Na^+deoxycholate) and transferred to a clean tube. This mixture was centrifuged for 10 minutes at 7000 x g in a Fisher microcentrifuge, the precipitate was resuspended in 0.5 ml RIPA, and this step was repeated. The suspension was centrifuged again, and the precipitate was resuspended in 30 ul cracking buffer (0.125 M Tris-Cl, pH 6.7, 10% glycerol, 5% mercaptoethanol, 2% SDS, 0.001% bromphenol blue). The mixture was placed in boiling water for 3 minutes to release the mutase from the S. aureus cells and then centrifuged for 5 minutes at 7000 x g in a Fisher microcentrifuge to remove the cells. The supernatant was applied to a SDS-polyacrlamide gel (see below).

Pulse-chase experiments

Labeling of the BRL cells for pulse-chase experiments was performed as described above for the immunoprecipitation of labeled mutase, except that in some experiments oxidative phosphorylation was blocked during the pulse using 2,4-dinitrophenol. After the cells had been incubated in 8 ml Eagle's minimal media without leucine for 30 minutes, 0.64 ml of 50 mM DNP in 0.1 M Tris base (pH 7.0) (DNP/Tris) was added to each dish for the remainder of the hour. After this media had been removed, 4ml Eagle's minimal medium minus leucine, 0.2 ml [^{3}H]leucine (47 Ci/mmol), and 0.32 ml of

DNP/Tris were added to the cells. After the 2 hour incubation period, the chase was performed by removing the media, rapidly rinsing the cells with 4 ml Eagle's minimal media without leucine, and then incubating the cells at 37°C in 4 ml Eagle's minimal medium without leucine with 0.2 ml of 0.02 mM unlabeled L-leucine for the desired chase time. After removing the medium and stopping the chase with 5 ml of cold phosphate-buffered saline, the cells were lysed with NETS-leu as above. The lysates were treated, immunoprecipitated, and prepared for application to the gel as described above.

In experiments in which the chase was performed in the presence of DNP, 0.32 ml DNP/Tris was included in the chase medium.

Growth of BRL cells in 2,4-dinitrophenol

Experiments were performed to determine whether incubation of BRL cells in the presence of DNP for at least 5 hours affects their ability to translate and process mutase. In one experiment, cells were incubated for 3 hours in 8 ml Dulbecco's modified medium and 0.64 ml DNP/Tris at 37°C. This medium was removed and the cells were labeled for 2 hours in the presence of DNP and chased in the absence of DNP as described above. To assess the ability of BRL cells to translate new mutase, cells were incubated for 5 hours in 8 ml Dulbecco's modified medium and 0.64 ml DNP/Tris before labelling them in the presence and absence

of DNP as described above.

Electrophoresis and visualization of radioactive mutase

The aliquots (~30 ul) containing immunoprecipitated mutase were applied to a 7.5% SDS-polyacrylamide slab gel (0.75 mm) (67). After electrophoresis (67) the gel was placed in a Coomassie brilliant blue solution for 30 minutes at 37°C for staining, then destained in a 10% acetic acid/10% methanol solution overnight. The gel was then placed in water for 15 minutes, followed by a 1 hour soak in Autofluor, and then the gel was dried using a Bio-Rad Model 221 gel slab dryer. It was placed against Kodak X-Omat XAR-5 film in the dark at -100°C for 3-5 days and developed.

RESULTS

Recovery of methylmalonyl CoA mutase from BRL cells

Prior to these studies, there have been no reports of the recovery of mutase from *in situ* labeled cells. As lanes 1 and 2 in figure 4 show, after cultured BRL cells were incubated in medium containing L-[^{3}H]leucine for 2 hours, immunoprecipitation of the cell lysate isolated a single polypeptide (lane 1). That this band represents methylmalonyl CoA mutase was verified by its absence in lane 2, where 0.03 mg of unlabeled, previously purified human liver mutase was added to the lysate before the addition of mutase antiserum. Mutase is absent in lane 2 because the excess unlabeled enzyme effectively competes for mutase antibody, preventing the endogenous labeled mutase from being immunoprecipitated. The arrow shows the position of the purified unlabeled human liver mutase on the Coomassie blue stained gel, corresponding exactly to the position on the fluorogram of labeled protein.

Discovery of the precursor form of mutase in in situ labeled cells

Because DNP has been shown to inhibit the translocation of other cytoplasmically synthesized mitochondrial proteins, it was used in this study in the hope of identifying a precursor form of mutase. Figure 5 shows that if the 2 hour

Figure 4. Recovery of mutase from BRL cells. BRL cells were labelled with [^{3}H]leucine for 2 hours. Immunoprecipitated mutase was recovered in the absence (lane 1) and presence (lane 2) of unlabeled mutase in the cell lysate. The arrow indicates the position of the unlabeled mutase on the Coomassie blue stained gel.

Figure 4. (legend on preceding page)

pulse is conducted in the presence of 4 mM DNP, the recovered protein runs more slowly on the gel (lane 2) than mutase from cells pulsed in the absence of DNP (lane 1). To determine if this more slowly moving band is indeed immunochemically related to methylmalonyl CoA mutase, experiments were performed in which the 2 hour pulse in the presence of DNP is followed by a 7 minute chase period in which both label and DNP are absent from the cell culture medium (figure 6). In lane 1, two bands can be visualized on the fluorogram, a faster moving band in the same position as authentic mutase (figure 4, lane 1) and the Coomassie blue stained band representing unlabeled purified human liver mutase (arrow, figure 6), and a slower moving band. When unlabeled purified mutase is added to the cell lysate before the addition of mutase antiserum, both of the bands disappear (figure 6, lane 2), verifying the immunochemical similarity between both polypeptides. Because DNP and other uncouplers of oxidative phosphorylation have been shown previously to inhibit the transformation of precursor cytoplasmic proteins to mature mitochondrial proteins (53,59,60,65), these data suggest that the more slowly moving (larger) band represents a cytoplasmic precursor to mature mutase. This precursor is about 3000 daltons larger than the mature mutase enzyme based on comparison with the migration of standard proteins (data not shown).

Figure 5. Recovery of pre-mutase from pulsed BRL cells. BRL cells were labelled with [^{3}H]leucine for 2 hours in the absence (lane 1) and presence (lane 2) of DNP.

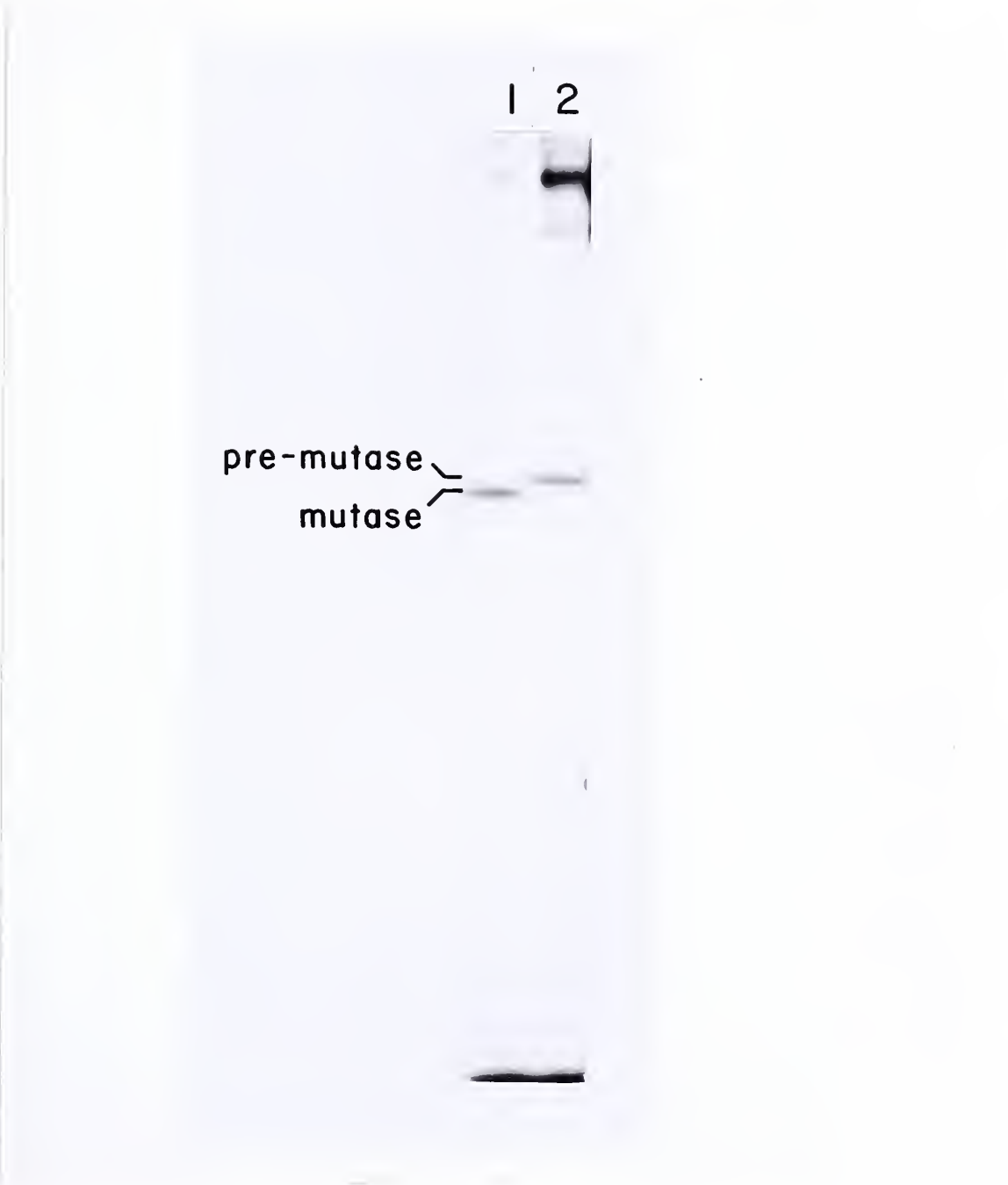


Figure 5. (legend on preceding page)

Figure 6. Immunochemical similarity between pre-mutase and mutase recovered from BRL cells. BRL cells were labeled with [^{3}H]leucine for 2 hours in the presence of DNP and then chased for 7 minutes in the absence of DNP. Immunoprecipitated protein was recovered in the absence (lane 1) and presence (lane 2) of unlabeled mutase in the cell lysate. The arrow indicates the position of the unlabeled mutase on the Coomassie blue stained gel.

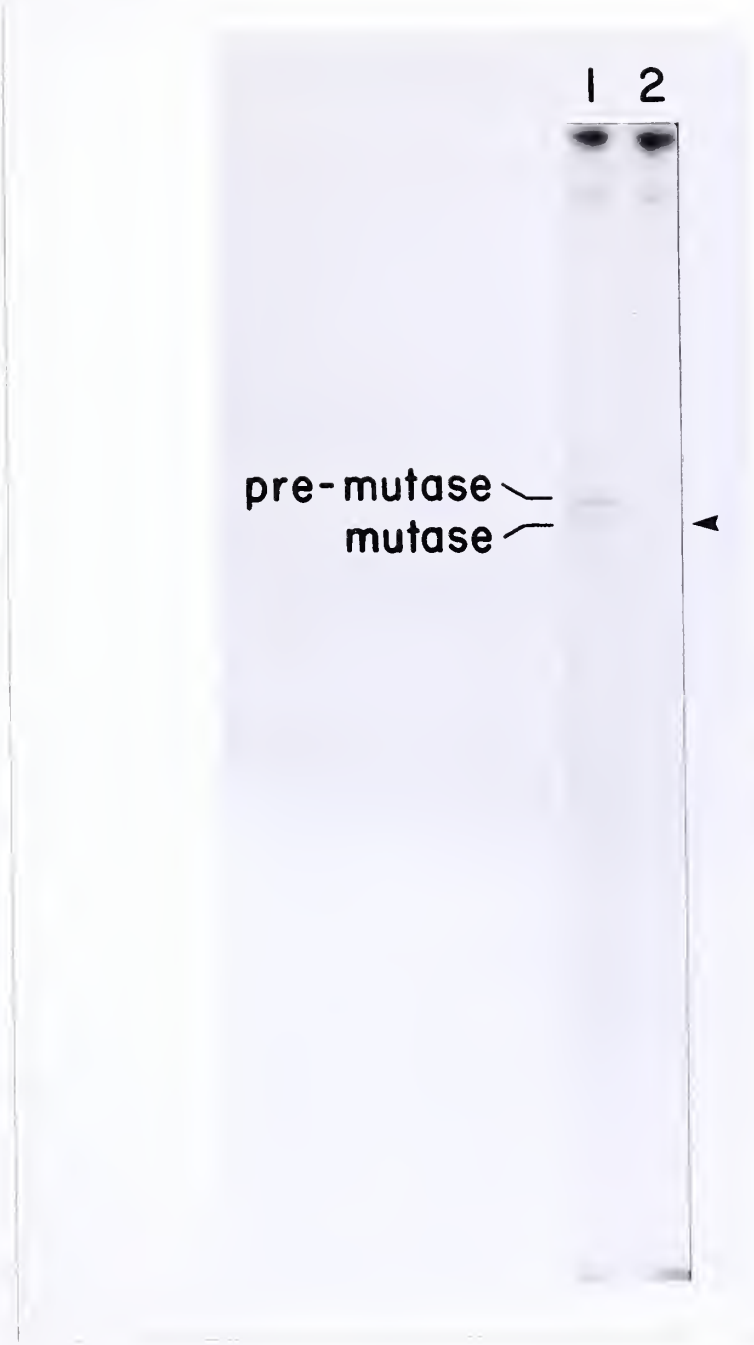


Figure 6. (legend on preceding page)

The larger form of mutase can be chased into the mature mutase band

The hypothesis that the larger form of methylmalonyl CoA mutase is a precursor to the form immunoprecipitated from labeled cells is supported by the experiment shown in figure 7A. BRL cells were pulsed with [^{3}H]leucine for 2 hours in the presence of DNP and then chased with unlabeled leucine in the absence of DNP for intervals ranging from zero to 12 minutes before the medium was removed and the cells lysed. Lanes 1 and 2 represent the 2 hour pulse in the absence and presence of DNP, respectively, without a chase. In lane 2, all of the radioactivity is associated with the pre-mutase band. After 2 minutes of chase (lane 3) a band becomes visible at the position corresponding to mature mutase. With longer chase times, this band becomes more intense, as the more slowly moving band representing pre-mutase becomes less intense (lanes 4-7), such that by 12 minutes of chase the pre-mutase band has almost completely disappeared. Hence, the radioactivity in the pre-mutase protein is incorporated into mature mutase protein over time, as soon as the block to conversion (i.e. DNP) is removed. From figure 7A it is qualitatively clear that after 6 minutes of chase the pre-mutase band is more intense than the mature mutase band, and after 9 minutes of chase the band representing mature mutase is more intense than the band in the position of pre-mutase. These results allow us to estimate that the $t_{1/2}$ for conversion of pre-mutase

Figure 7. A. Chase of pre-mutase into mature mutase. BRL cells were labelled with [^{3}H]leucine for 2 hours in the absence (lane 1) and presence (lanes 2-7) of DNP. The cells were chased for 0 minutes (lanes 1 and 2), 2 minutes (lane 3), 4 minutes (lane 4), 6 minutes (lane 5), 9 minutes (lane 6) and 12 minutes (lane 7) in the absence of DNP.

B. Chase of pulsed BRL cells in the presence of DNP. BRL cells were labelled with [^{3}H]leucine for 2 hours in the absence (lanes 1 and 2) and presence (lanes 3-6) of DNP. The cells in lanes 3-6 were chased in the presence of DNP for 0 hours (lane 3), 1 hour (lane 4), 3 hours (lane 5) and 5 hours (lane 6). The cells in lanes 1 and 2 were chased in the absence of DNP for 0 hours (lane 1) and 5 hours (lane 2).

Figure 7. (legend on preceding page)

protein to mature mutase enzyme is between 6 and 9 minutes after the removal of DNP.

Chase of pulsed BRL cells with DNP present

Based on the reports of studies with OTCase and aspartate aminotransferase (64.65), one would expect to witness rapid degradation of the mutase precursor if DNP is used throughout the chase period to keep the mitochondrial translocation/processing system blocked. Figure 7B shows, however, that the band representing mutase precursor (lanes 3-6) remains strong for as long as 5 hours of chase in the absence of label. Qualitatively, the pre-mutase band does not appear to show any significant decrease in intensity until 5 hours (lane 6). In no lane could there be seen any density associated with mature mutase (compare with lanes 1 and 2) or apparent degradation products of unprocessed pre-mutase.

The ability of BRL cells to process pre-mutase and translate new mutase after exposure to DNP for 5 hours was assessed. Figure 8 shows that BRL cells can process labeled pre-mutase after 5 hours of growth in medium containing 4 mM DNP (lane 1) and that they are capable of translating new mutase if the cells are labeled after they have been incubated for 5 hours in this medium (lanes 2 and 3). Hence, it is unlikely that the persistence of a band representing pre-mutase after 5 hours of chase in the presence of DNP can be attributed to a DNP-related breakdown

of the cells' metabolic processes; there appears to be no change in the ability of the BRL cell to translate and process mutase under these conditions.

Figure 8. Affect of DNP on the processing and translation of mutase. In the experiment shown in lane 1, BRL cells were incubated in medium containing 4 mM DNP for 3 hours and then labeled with [^{3}H]leucine for 2 hours in the presence of DNP. The cells were then chased for 1 hour with unlabeled leucine in the absence of DNP. In another experiment, BRL cells were incubated for 5 hours in medium containing 4 mM DNP and then labeled for 2 hours with [^{3}H]leucine in the absence (lane 2) or presence (lane 3) of DNP.

Figure 8. (legend on preceding page)

DISCUSSION

It is clear from the introductory review of cytoplasmically synthesized mitochondrial proteins that the details of their translocation and processing are not yet well worked out. Although there are many common elements among the results which various workers have obtained, some of the evidence accumulated so far is conflicting and it is unclear whether results from one type of organism (e.g. yeast) can be generalized to another (e.g. mammals). The work presented in this thesis is intended to add to the knowledge of the biology of mitochondrial proteins synthesized in the cytoplasm and also to contribute to the understanding of the biosynthesis and processing of methylmalonyl CoA mutase.

The autosomal recessive inheritance pattern of methylmalonic acidemia in the mut mutant class implies that methylmalonyl CoA mutase must be coded on nuclear DNA rather than mitochondrial DNA. One of the most important results of this work is that methylmalonyl CoA mutase has been shown to be synthesized in a larger precursor form which cannot be processed in the presence of an uncoupler of oxidative phosphorylation, presumably because the uncoupler deprives the inner membrane of energy required for translocation (figure 5). Hence, methylmalonyl CoA mutase can be added to the growing list of proteins which have been shown to be synthesized cytoplasmically as a precursor and processed to

a smaller, mature mitochondrial form.

Although the processing of *in vitro* translation system products by isolated mitochondria has been taken as evidence that free precursors can be imported and processed *in vivo*, there had been no direct reports prior to the undertaking of this work that free precursors could be imported by mitochondria in intact cells. Other studies had only shown that precursor accumulated in the extramitochondrial compartment if translocation was blocked. Figures 6 and 7A conclusively show that precursor accumulated in the presence of DNP can subsequently be chased to mature enzyme when DNP is removed from intact BRL cells 2 hours later. Hence, the *in vitro* findings are substantiated by these *in vivo* data. After these studies had been completed, Schatz and coworkers reported similar results for cytochrome b_2, cytochrome c_1 and the beta subunit of the F_1-ATPase using pulsed yeast spheroplasts in the presence of CCCP (60,68).

As discussed earlier, a few groups have determined half-times for translocation and processing by chasing pulse-labeled cells. The method used in this study (figure 7A) differs in that the BRL cells were pulsed in the presence of DNP. Hence, before chasing in the absence of label and DNP, precursor accumulated without the opportunity to be converted to mature enzyme. Other workers have not used such a translocation inhibitor during the pulse in their experiments to calculate the $t_{1/2}$ for conversion. To draw reliable conclusions about the half-time for conversion

with the method used in this work, two conditions must be met: 1) there is no significant degradation of accumulated precursor during the pulse period; and, 2) there is not a significant period of time required after the removal of DNP for its effects to be reversed. The experiment shown in figure 7B, which demonstrated that there was no significant degradation of precursor over many hours, shows that the first condition is satisfied. Fulfillment of the second condition is established by examining lane 3 in figure 7A; at 2 minutes of chase there is already some conversion of pre-methylmalonyl CoA mutase to mature mutase. indicating that any lag-time required for the effects of DNP to be reversed must be less than 2 minutes.

The results in figure 7A also show that the $t_{1/2}$ for the conversion of pre-mutase to mature mutase is between 6 and 9 minutes. This time is a bit longer than the half-lives reported for OTCase, CPS or aspartate aminotransferase (63,64,65). The reasons for this small difference may be related to factors such as size, charge and tertiary configuration influencing the processing of protein precursors. However, the half-life for processing of precursor to mature mitochondrial protein has been studied for only 4 proteins; it may be found after future study that the similarity in half-lives (i.e. minutes) is more striking than the differences between them.

The most unexpected discovery from this work is shown in figure 7B. Instead of undergoing rapid degradation when

the cells are chased in the presence of DNP, the labeled mutase precursor can be immunoprecipitated for at least 5 hours without signs of significant degradation. Therefore, if the presumptive degradation follows first order kinetics, the $t_{1/2}$ for the disappearance of pre-mutase is longer than 5 hours. This is very different from the $t_{1/2}$ for the disappearance of pre-CPS from rat liver explants in which processing was blocked (2-3 minutes) (64), or the $t_{1/2}$ for the disappearance of aspartate aminotransferase from individual rat hepatocytes in which translocation was blocked (~5 minutes) (65). Reid and Schatz, in a report published after the completion of this work, monitored the presence of labeled pre-beta subunit of the F_1-ATPase of mutant yeast cells incubated with CCCP and $^{35}SO_4^{2-}$ for as long as 7.5 hours (69). However, these cells were not chased; the labeled precursor may simply have represented continually synthesized protein. Therefore, conclusions about precursor lifetime based on such experiments may be invalid. Hence, of the few reports which describe the $t_{1/2}$ for precursor degradation, mutase is the first case in which degradation does not occur within a few minutes. We can only speculate about the reasons for this. If an accumulated protein precursor can be recognized as an abnormal protein, its rapid degradation can be explained (66). However, if, in general, accumulated precursors are recognized as abnormal, the reason why pre-mutase is not similarly recognized is unclear. Possibly its charge and tertiary

structure are very similar to that of the mature form. More interestingly, perhaps the methylmalonyl CoA mutase precursor form is not exposed to the degrading system. The precursor may be within the mitochondrion, protected from a cytoplasmic degrading system, unable to be translocated and processed properly because of the presence of DNP. This work has not identified an intermediate form for mutase. so to postulate that DNP does not inhibit processing to an intermediate form, which is protected from the degradative process but unable to form mature enzyme, is unwarranted.

While new information has been generated from this work, it has also provided the impetus for further study of mutase in its cytoplasmic and mitochondrial forms. For instance, it would be very helpful to know where the non-degraded mutase precursor is located in cells which are exposed to DNP. Attempts to determine this are presently ongoing. In addition, it would be interesting to know if the accumulation of precursor in DNP-exposed cells exerts negative feedback control on the further synthesis of pre-mutase. This can be separated out from any confounding degradation process by using a different radioactive species (e.g. [^{14}C]leucine) to label BRL cells during the "chase" period in the presence of DNP. The relative contributions of ^{3}H and ^{14}C to the total radioactivity present in immunoprecipitated pre-mutase could be used to calculate the rates of pre-mutase synthesis during the pulse and chase periods. Alternatively, unlabeled leucine could be used to

accumulate precursor in the presence of DNP, followed by incubation with DNP and labeled leucine to assess additional accumulation.

In addition to contributing to our knowledge of the biology of cytoplasmically synthesized mitochondrial proteins, the study of methylmalonyl CoA mutase is important to understanding the biochemistry of the apoenzyme mutant (mut) class of methylmalonic acidemia. It has been established that the mutase enzyme of the mut^- subgroup does not bind cofactor as well as normal mutase, providing a rationale for the disease state in these patients. For the mut^0 mutants, however, a number of explanations can be advanced, including frameshift mutations, regulatory gene mutations, or defects in mRNA processing. With the new knowledge that mutase is indeed synthesized in the cytoplasm in a precursor form, an additional explanation for the existence of the mut^0 group can be proposed. Perhaps a mutation in the signal sequence of pre-mutase does not allow proper recognition of the precursor by the mitochondrial outer membrane, or translocation across the inner membrane, or proper processing protease recognition, such that a mature, active mutase enzyme is never formed. This explanation could account for the CRM^- subgroup of mut^0 mutants if these aberrantly synthesized precursors were degraded rapidly rather than accumulated, but the results from this work do not support such degradation in Buffalo rat liver cells. It seems more likely that a mutation in

the signal sequence of precursor mutase might result in a CRM^+ mutant. Even with a drastically altered signal sequence, small relative to the size of the entire precursor form, immunochemically cross-reacting material should be present. Clearly, this work must be extended to human fibroblasts from normals and *mut*^{0} patients.

That such a mutation must be in the precursor and not in a mitochondrial outer membrane receptor, in the translocation apparatus, or in a processing protease is supported by two lines of evidence. First, it seems unlikely that a different system exists for import, translocation, and processing of all the different mitochondrial proteins synthesized in the cytoplasm. If overlap existed such that more than one protein could utilize the same systems, a patient with a mutation in one processing system would have many pleiotropic metabolic disorders. This is not the case in methylmalonic acidemia. Second, and more convincing, it has been shown repeatedly that *mut*$^-$ and *mut*^{0} mutant cells are not complementary when fused with each other. They are both groups within the *mut* complementation class. The *mut*$^-$ defect consists of a mutase apoenzyme with abnormally low affinity for cofactor; the most likely explanation for this is a mutation in the structural gene for mutase at the cofactor binding site. Since *mut*$^-$/*mut*^{0} heterokaryons are non-complementary, the *mut*^{0} group must also be characterized by a mutation in the mutase apoenzyme structural gene. If a defect in a

mitochondrial system explained the *mut*^{0} group, then *mut*^{0}/*mut*$^{-}$ heterokaryons ought to be complementary-- the *mut*^{0} cells could take advantage of the *mut*$^{-}$ cells' normal mitochondria. I propose, then, the following explanations for the *mut*^{0} group of mutants based on this work accomplished with BRL cells: The CRM^{-} subgroup represents a mutant in which the mutase precursor is not synthesized or is drastically altered such that it is either not recognized by antiserum or degraded. The CRM^{+} subgroup represents either a small mutation at the active site of mutase such that it is not degraded and is still immunochemically recognizable, or it represents a mutation in the signal sequence of pre-mutase such that immunochemically reactive precursor is accumulated but cannot be imported or converted to its mature form by mitochondria.

In summary, it has been shown using cultured BRL cells that methylmalonyl CoA mutase is made *in vivo* as a precursor which can be converted to mature mitochondrial mutase with a $t_{1/2}$ of 6-9 minutes. In contrast to other cytoplasmically synthesized mitochondrial proteins studied, pre-mutase is not degraded rapidly if translocation within the mitochondrion is blocked with an uncoupler of oxidative phosphorylation. The implications of these findings with respect to the biosynthesis of mitochondrial proteins and the *mut* mutant class of methylmalonic acidemia have been discussed. I propose that the CRM^{+} subgroup of this class may represent a mutation in the signal sequence of pre-

mutase such that precursor is accumulated but cannot be imported or converted to its mature form by mitochondria.

BIBLIOGRAPHY

1. Rosenberg, L.E. 1978. "Disorders of propionate, methylmalonate, and cobalamin metabolism in The Metabolic Basis of Inherited Diseases, J.B. Stanbury et al (ed.) New York: McGraw-Hill Book Co. pp. 411-429.

2. Flavin, M., Ortiz, P.J., and Ochoa, S. 1955. Metabolism of propionic acid in animal tissues. Nature 176: 823-826.

3. Mazumder, R., Sasakawa, T., and Ochoa, S. 1963. Metabolism of propionic acid in animal tissues X. Methylmalonyl coenzyme A mutase holoenzyme. J. Biol. Chem. 238: 50-53.

4. Cannata, J.J.B., Focesi, A., Mazumder R., Warner, R.C., and Ochoa, S. 1965. Metabolism of propionic acid in animal tissues XII. Properties of mammalian methylmalonyl coenzyme A mutase. J. Biol. Chem. 240: 3249-3257.

5. Fenton, W.A., Hack, A.M., Willard, H.F., Gertler, A., and Rosenberg, L.E. 1982. Purification and properties of methylmalonyl coenzyme A mutase from human liver. Arch. Biochem. Biophys. 214: 815-823.

6. Hegre, C.S., Miller S.J., and Lane, M.D. 1962. Studies on methylmalonyl isomerase. Biochim. Biophys. Acta 56: 538-544.

7. Kellermeyer, R.W. and Wood, H.G. 1962. Methylmalonyl isomerase: A study of the mechanism of isomerization. Biochemistry 1: 1124-1131.

8. Smith, R.M. and Monty, K.J. 1959. Vitamin B_{12} and propionate metabolism. Biochem. Biophys. Res. Commun. 1: 105-109.

9. Gurnani, S., Mistry, S.P., and Johnson, B.C. 1960. Function of vitamin B_{12} in methylmalonate metabolism 1. Effect of a cofactor form of B_{12} on the activity of methylmalonyl-CoA isomerase. Biochim. Biophys. Acta 38: 187-188.

10. Stern, J.R. and Friedman, D.L. 1960. Vitamin B_{12} and methylmalonyl CoA isomerase. Biochem. Biophys. Res. Commun. 2: 82-87.

11. Youngdahl-Turner, P., Rosenberg, L.E., and Allen, R.H. 1978. Binding and uptake of transcobalamin II by

human fibroblasts. J. Clin. Invest. 61: 133-141.

12. Wang, F.K., Koch, J., and Stokstad, E.L.R. 1967. Folate coenzyme pattern, folate linked enzymes and methionine biosynthesis in ratliver mitochondria. Biochem. Z. 346: 458-466.

13. Frenkel, E.P. and Kitchens, R.L. 1975. Intracellular localization of hepatic propionyl CoA carboxylase and methylmalonyl CoA mutase in humans and normal and vitamin B_{12} deficient rats. Br. J. Haemat. 31: 501-513.

14. Mahoney, M.J., Hart, A.C., Steen, V.D., and Rosenberg, L.E. 1975. Methylmalonicacidemia: Biochemical heterogeneity in defects of 5'-deoxyadenosylcobalamin synthesis. Proc. Natl. Acad. Sci. 72: 2799-2803.

15. Vitols, E., Walker, G.A., and Huennekens, F.M. 1966. Enzymatic conversion of vitamin B_{12} to a cobamide coenzyme, alpha-(5,6-dimethylbenzimidazolyl)deoxy-adenosylcobamide (adenosyl-B_{12}) J. Biol. Chem. 241: 1455-1461.

16. Barness, L.A., Young, D., Mellman, W.J., Kahn, S.B., and Williams, W.J. 1963. Methylmalonate excretion in a patient with pernicious anemia. N. Engl. J. Med. 268: 144-146.

17. Cox, E.V. and White, A.M. 1962. Methylmalonic acid excretion: Index of vitamin-B_{12} deficiency. Lancet 2: 853-855.

18. Oberholzer, V.G., Levin, B., Burgess E.A., and Young, W.F. 1967. Methylmalonic Aciduria: An inborn error of metabolism leading to chronic metabolic acidosis. Arch. Dis. Childh. 42: 492-504.

19. Stokke, O., Eldjarn, L., Norum, K.R., Steen-Johnsen, J., and Halvorsen, S. 1967. Methylmalonic Acidemia: A new inborn error of metabolism which may cause fatal acidosis in the neonatal period. Scand. J. Clin. Lab. Invest. 20: 313-328.

20. Lindblad, B., Lindblad, B.S., Olin, P., Svanberg, B., and Zetterstrom, R. 1968. Methylmalonic Acidemia: A disorder associated with acidosis, hyperglycinemia, and hyperlactatemia. Acta Paediat. Scand. 57: 417-424.

21. Rosenberg, L.E., Lilljeqvist, A., and Hsia, Y.E. 1968. Methylmalonic Aciduria: An inborn error of metabolism leading to metabolic acidosis, long-chain ketonuria and intermittent hyperglycinemia. N.

Engl. J. Med. 278: 1319-1322.

22. Gruskay, J.A. 1981. Inhibition of mitochondrial carbamyl phosphate synthetase in rat and human liver by acyl CoA esters: A possible mechanism for hyperammonemia in the inherited organic acidemias. Thesis, Yale University School of Medicine.

23. Kolvraa, S. 1979. Inhibition of the glycine cleavage system by branched-chain amino acid metabolites. Pediat. Res. 13: 889-893.

24. Rosenberg, L.E., Lilljeqvist, A., and Hsia, Y.E. 1968. Methylmalonic Aciduria: Metabolic block localization and vitamin B_{12} dependency. Science 162: 805-807.

25. Lindblad, B., Lindstrand, K., Svanberg, B. and Zetterstrom, R. 1969. The effect of cobamide coenzyme in methylmalonic acidemia. Acta Paediat. Scand. 58: 178-180.

26. Morrow, G., Barness, L.A., Cardinale, G.J., Abeles, R.H., and Flaks, J.G. 1969. Congenital methylmalonic acidemia: Enzymatic evidence fortwo forms of the disease. Proc. Natl. Acad. Sci. 63: 191-197.

27. Rosenberg, L.E., Lilljeqvist, A., Hsia, Y.E. and Rosenbloom, F.M. 1969. Vitamin B_{12} dependent methylmalonicaciduria: Defective B_{12} metabolism in cultured fibroblasts. Biochem. Biophys. Res. Commun. 37: 607-614.

28. Kaye, C.I., Morrow, G., and Nadler, H.L. 1974. In vitro "responsive" methylmalonic acidemia: A new variant. J. Pediat. 85: 55-59.

29. Fenton, W.A. and Rosenberg, L.E. 1981. The defect in the cbl B class of human methylmalonic acidemia: Deficiency of cob(I)alamin adenosyltransferase activity in extracts of cultured fibroblasts. Biochem. Biophys. Res. Commun. 98: 283-289.

30. Mudd, S.H., Levy, H.L. and Abeles, R.H. 1969. A derangement in B_{12} metabolism leading to homocystinemia, cystathionemia and methylmalonic aciduria. Biochem. Biophys. Res. Commun. 35: 121-126.

31. Mahoney, M.J., Rosenberg, L.E., Mudd, S.H., and Uhlendorf, B.W. 1971. Defective metabolism of vitamin B_{12} in fibroblasts from children with methylmalonicaciduria. Biochem. Biophys. Res.

Commun. 44: 375-381.

32. Mellman, I., Willard, H.F., Youngdahl-Turner, P., and Rosenberg, L.E. 1979. Cobalamin coenzyme synthesis in normal and mutant human fibroblasts: Evidence for a processing enzyme activity deficient in cbl C cells. J. Biol. Chem. 254: 11847-11853.

33. Gravel, R.A., Mahoney, M.J., Ruddle, F.H., and Rosenberg, L.E. 1975. Genetic complementation in heterokaryons of human fibroblasts defective in cobalamin metabolism. Proc. Natl. Acad. Sci. 72: 3181-3185.

34. Willard, H.F., Mellman, I.S., and Rosenberg, L.E. 1978. Genetic complementation among inherited deficiencies of methylmalonyl-CoA mutase activity: Evidence for a new class of human cobalamin mutant. Am. J. Hum. Genet. 30: 1-13.

35. Willard, H.F. and Rosenberg, L.E. 1977. Inherited deficiencies of human methylmalonyl CoA mutase activity: Reduced affinity of mutant apoenzyme for adenosylcobalamin. Biochem. Biophys. Res. Commun. 78: 927-934.

36. Morrow, G., Revsin, B., Clark, R., Lebowitz, J., and Whelan, D.T. 1978. A new variant of methylmalonic acidemia- defective coenzyme-apoenzyme binding in cultured fibroblasts. Clin. Chim. Acta 85: 67-72.

37. Willard, H.F. and Rosenberg, L.E. 1980. Inherited methylmalonyl CoA mutase apoenzyme deficiency in human fibroblasts: Evidence for allelic heterogeneity, genetic compounds, and codominant expression. J. Clin. Invest. 65: 690-698.

38. Kolhouse, J.F., Utley, C., Fenton, W.A., and Rosenberg, L.E. 1981. Immunochemical studies on cultured fibroblasts from patients with inherited methylmalonic acidemia. Proc. Natl. Acad. Sci. 78: 7737-7741.

39. Neupert, W. and Schatz, G. 1981. How proteins are transported into mitochondria. Trends Biochem. Sci. 6: 1-4.

40. Schatz, G. and Mason, T.L. 1974. The biosynthesis of mitochondrial proteins. Ann. Rev. Biochem. 43: 51-87.

41. Blobel, G. and Dobberstein, B. 1975. Transfer of proteins across membranes I. Presence of proteolytically processed and unprocessed nascent

immunoglobulin light chains in membrane-bound ribosomes of murine myeloma. J. Cell. Biol. 67: 835-851.

42. Blobel, G. and Dobberstein, R. 1975. Transfer of proteins across membranes II. Reconstitution of functional rough microsomes from heterologous components. J. Cell. Biol. 67: 852-862.

43. Suissa, M. and Schatz, G. 1982. Import of proteins into mitochondria. Translatable mRNA's for imported mitochondrial proteins are present in free as well as mitochondria-bound cytoplasmic polysomes. J. Biol. Chem. 257: 13048-13055.

44. Morita, T., Mori, M., Tatibana, M., and Cohen, P.P. 1981. Site of synthesis and intracellular transport of the precursor of mitochondrial ornithine transcarbamoylase. Biochem. Biophys. Res. Commun. 99: 623-629.

45. Chua, N. and Schmidt, G.W. 1978. Post-translational transport into intact chloroplasts of a precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase. Proc. Natl. Acad. Sci. 75: 6110-6114.

46. Maccecchini, M., Rudin, Y., Blobel, G., and Schatz, G. 1979. Import of proteins into mitochondria: Precursor forms of the extramitochondrially made F_1-ATPase subunits in yeast. Proc. Natl. Acad. Sci. 76: 343-347.

47. Gasser, S.M., Daum, G., and Schatz, G. 1982. Import of proteins into mitochondria. Energy-dependent uptake of precursors by isolated mitochondria. J. Biol. Chem. 257: 13034-13041.

48. Lewin, A.S., Gregor, I., Mason, T.L., Nelson, N., and Schatz, G. 1980. Cytoplasmically made subunits of yeast mitochondrial F_1-ATPase and cytochrome c oxidase are synthesized as individual precursors, not as polyproteins. Proc. Natl. Acad. Sci. 77: 3998-4002.

49. Mihara, K. and Blobel, G. 1980. The four cytoplasmically made subunits of yeast mitochondrial cytochrome c oxidase are synthesized individually and not as a polyprotein. Proc. Natl. Acad. Sci. 77: 4160-4164.

50. Cote, C., Solioz, M., and Schatz, G. 1979. Biogenesis of the cytochrome bc_1 complex of yeast mitochondria. A precursor form of the cytoplasmically made subunit V. J. Biol. Chem. 254: 1437-1439.

51. Maccecchini, M., Rudin, Y., and Schatz, G. 1979. Transport of proteins across the mitochondrial outer membrane. A precursor form of the cytoplasmically made intermembrane enzyme cytochrome c peroxidase. J. Biol. Chem. 254: 7468-7471.

52. Schmelzer, E. and Heinrich, P.C. 1980. Synthesis of a larger precursor for the subunit IV of rat liver cytochrome c oxidase in a cell-free wheat germ system. J. Biol. Chem. 255: 7503-7506.

53. Nelson, N. and Schatz, G. 1979. Energy-dependent processing of cytoplasmically made precursors to mitochondrial proteins. Proc. Natl. Acad. Sci. 76: 4365-4369.

54. Shore, G.C., Carignan, P. and Raymond, Y. 1979. *In vitro* synthesis of a putative precursor to the mitochondrial enzyme, carbamyl phosphate synthetase. J. Biol. Chem. 254: 3141-3144.

55. Conboy, J.G., Kalousek, F., and Rosenberg, L.E. 1979. *In vitro* synthesis of a putative precursor of mitochondrial ornithine transcarbamoylase. Proc. Natl. Acad. Sci. 76: 5724-5727.

56. Mori, M., Miura, S., Tatibana, M., and Cohen, P.P. 1980. Characteristics of a protease apparently involved in processing of pre-ornithine transcarbamylase of rat liver. Proc. Natl. Acad. Sci. 77: 7044-7048.

57. Conboy, J.G. and Rosenberg, L.E. 1981. Posttranslational uptake and processing of *in vitro* synthesized ornithine transcarbamoylase precursor by isolated rat liver mitochondria. Proc. Natl. Acad. Sci. 78: 3073-3077.

58. Conboy, J.G., Fenton, W.A., and Rosenberg, L.E. 1982. Processing of pre-ornithine transcarbamylase requires a zinc-dependent protease localized to the mitochondrial matrix. Biochem. Biophys. Res. Commun. 105: 1-7.

59. Kolansky, D.M., Conboy, J.G., Fenton, W.A., and Rosenberg, L.E. 1982. Energy-dependent translocation of the precursor of ornithine transcarbamylase by isolated rat liver mitochondria. J. Biol. Chem. 257: 8467-8471.

60. Reid, G.A., Yonetani, T., and Schatz, G. 1982. Import of proteins into mitochondria. Import and maturation of the mitochondrial intermembrane space enzymes cytochrome b_2 and cytochrome c peroxidase in

intact yeast cells. J. Biol. Chem. 257: 13068-13074.

61. Daum, G., Gasser, S.M., and Schatz, G. 1982. Import of proteins into mitochondria. Energy-dependent two-step processing of the intermembrane space enzyme cytochrome b_2 by isolated yeast mitochondria. J. Biol. Chem. 257: 13075-13080.

62. Ohashi, A., Gibson, J., Gregor, I., and Schatz, G. 1982. Import of proteins into mitochondria. The precursor of cytochrome c_1 is processed in two steps, one of them heme-dependent. J. Biol. Chem. 257: 13042-13047.

63. Raymond, Y. and Shore, G.C. 1981. Processing of the precursor for the mitochondrial enzyme, carbamyl phosphate synthetase. Inhibition by para-aminobenzamidine leads to very rapid degradation (clearing) of the precursor. J. Biol. Chem. 256: 2087-2090.

64. Mori, M., Morita, T., Ikeda, F., Amaya, Y., Tatibana, M., and Cohen, P.P. 1981. Synthesis, intracellular transport, and processing for mitochondrial ornithine transcarbamylase and carbamoyl-phosphate synthetase I in isolated hepatocytes. Proc. Natl. Acad. Sci. 78: 6056-6060.

65. Jaussi R., Sonderegger, P., Fluckiger, J., and Christen, P. 1982. Biosynthesis and topogenesis of aspartate aminotransferase isoenzymes in chicken embryo fibroblasts. The precursor of the mitochondrial isoenzyme is either imported into mitochondria or degraded in the cytosol. J. Biol. Chem. 257: 13334-13340.

66. Goldberg, A.L. and St. John, A.C. 1976. Intracellular protein degradation in mammalian and bacterial cells: Part 2. Ann. Rev. Biochem. 45: 747-803.

67. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

68. Reid, G.A. and Schatz, G. 1982. Import of proteins into mitochondria. Extramitochondrial pools and post-translational import of mitochondrial precursors *in vivo*. J. Biol. Chem. 257: 13062-13067.

69. Reid, G.A. and Schatz, G. 1982. Import of proteins into mitochondria. Yeast cells grown in the presence of carbonyl cyanide *m*-chlorophenylhydrazone

accumulate massive amounts of some mitochondrial precursor polypeptides. J. Biol. Chem. 257: 13056-13061.

YALE MEDICAL LIBRARY

Manuscript Theses

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Yale Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS	DATE